THE EFFECT OF CYCLIC ADENOSINE MONOPHOSPHATE (cAMP) MODULATORS ON THE ACTIVITY OF SELECTED ANTIMALARIAL DRUGS

By

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Dedication

I dedicate this work to my loving parents, Isabella Wamuyu and Agostino Mwangi.

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Abstract

Malaria is one of the major global health problems with high mortality rates especially in children below five years of age. The interventions in place to control the disease worldwide include vector control and chemotherapy. Chemotherapy has developed crippling limitations due to malaria drug resistance to the effective, available and affordable anti-malarials. The emergence of parasite resistance has limited the armory of effective anti-malarials. Resistance is developing faster than the development of new and effective anti-malarials. This has raised a need of therapy optimization using the already existing anti-malarials. Combination therapy has been shown to effectively delay the onset of resistance and improve the efficacy of two or more anti-malarials when in combination. The objective of this study was to establish the activity of cAMP modulators on a few selected anti-malarials *in vitro*. The cAMP modulators were tested in combination with chloroquine, quinine, mefloquine, amodiaquine and doxycycline against *Plasmodium falciparum in vitro* against chloroquine sensitive strain (D6) and the chloroquine resistant strain (W2). Parasite susceptibility testing was carried out using semi automated micro dilution technique. The Inhibitory Concentration at 50% (IC_{50}) was calculated for each drug and for the drugs in fixed combination (1:1, 1:3, 3:1, 1:4, 4:1, 1:5). These data were used to calculate the Fractional Inhibitory Concentration at 50% (FIC_{50}) and to plot isobolograms. Quinine-AC inhibitors combination assays showed synergistic interactions in all the levels of concentration ratios against both the D6 and W2. The other anti-malarials-AC inhibitor combination assays showed a range of response, additivity, synergism and antagonism at different levels of concentration ratios. Anti-malarials-AC activators mainly exhibited antagonistic interactions. These findings suggest that quinine-AC inhibitors combination should be considered for evaluation as possible new anti-malarial combinations.

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List of abbreviations

9-cyclo-	9 Cyclopentyladenine
A 166 -	Sp-adenosine 3'5' cyclic monophosphorothiate triethylammonium salt
AC -	Adenylyl cyclase
ADP -	Adenosine diphosphate
ATP -	Adenosine triphosphate
AQN -	Amodiaquine
cAMP -	cyclic Adenosine 3′5′ Monophosphate
CDO -	cAMP response decoy oligonucleotide
cGMP -	Cyclic guanine monophosphate
CMS -	Complete media with serum
CRE -	cAMP response element
CSP -	Circumsporozoite Protein
CQN -	Chloroquine
DHFR -	Dihydrofolate Reductase
DHPS -	Dihydropteroate Sythetase
D0689 -	2′5′ Dideoxyadenosine 3′ monophosphate
DMSO -	Dimethyl sulfoxide
DOXY -	Doxycycline
FAS -	Fatty Acid Synthetase
FIC_{50} -	Fractional inhibitory concentrations at fifty percent
GDP -	Guanine Diphosphate
G_i -	Guanine inhibitory

G- Protein- Guanine nucleotide binding proteins

G_s - Guanine stimulatory

GTP - Guanine triphosphate

HCT - Hematocrit

IC_{50} - Inhibitory Concentrations at 50 percent

Kb - Kilo base pair

KDa - Kilo dalton

MQN - Mefloquine

NPP - New permeation pathway

PfAC - *Plasmodium falciparum* adenylyl cyclase

PfCRT - *Plasmodium falciparum* Chloroquine resistant trait

Pfmdr - *Plasmodium falciparum* multi-drug resistance

PKA - Protein Kinase A

PKA-C- cAMP dependent Protein Kinase A

pLDH - Plasmodium lactate Dehydrogenase

PPM - Parasite plasma membrane

PVM - Parasitophorous vacuolar membrane

QN - Quinine

SP - Sulfadoxine-Pyrimethamine

TRAP - Thrombospondin related adhesive protein

TVM - Tubovesicular membrane

WRAIR - Walter Reed Army Institute of Research

CHAPTER ONE

1.1 INTRODUCTION AND LITERATURE REVIEW

1.2 Background

Malaria is one of the most ancient unresolved afflictions of human kind which affects over half a billion people resulting in 1.5 million to 2.7 million deaths yearly especially children below five years of age where the disease is hyper endemic (Suh *et al.*, 2004). Today malaria is considered as a disease of the poor and undeveloped countries. The inability of many countries to fund expensive campaigns and anti-malarial treatment raises the need of new tools that are highly effective and affordable to be put in place (Weatherall *et al.*, 2002). The annual economic growth in countries with high transmission has been found to be lower than in those countries without malaria (WHO, 2001).

The emergence of resistance can be attributed to production of resistant mutants and a subsequent selection process whereby the presence of anti-malarials in the human host is an advantage for their survival. This leads to preferential transmission and spread of resistance (White, 2004). This has resulted in development of resistance to the commonly used anti-malarials. They have been rendered ineffective, hence the need to develop affordable and effective alternatives.

Several approaches are being made towards drug discovery (Rosenthal, 2003). Research studies to understand the mechanisms of cellular growth and differentiation in an effort to identify potential targets are on the increase (Waters and Geyer, 2003). This

has resulted in a better understanding of the molecular mechanisms controlling the progression of the life cycle of malaria species especially *Plasmodium falciparum*.

Cyclic adenosine monophosphate (cAMP) is a second messenger involved in the regulation of development of several microorganisms. It acts as a signal for the aggregation and differentiation of cells in multi-cellular organisms (Syin *et al*., 2001). Other than its key role as a second messenger, it modulates a myriad of physiological processes in a cell (Francis and Corbin, 1999). The molecular machinery controlling cAMP production, degradation and sensitivity of Plasmodia is the same as that of higher eukaryotes (Beraldo *et al*., 2005).

Intra-erythrocytic development of plasmodia parasites depend on a series of complex signaling pathways in which second messengers, for example, cAMP and calcium play a key role (Elford *et al.,* 1997). cAMP has been implicated in the regulation of sexual differentiation in *P. falciparum* and has been shown to have cytotoxic effects under certain conditions. The fundamental regulatory role that the cyclic nucleotides play in biological processes may be used in designing new anti-malarial strategies (Sheppard *et al*., 1981).

The purpose of this work was to determine the activity of cAMP modulators on selected anti-malarials in *Plasmodium falciparum.*

1.2 LITERATURE REVIEW

1.2.1 Etiology of Malaria

Human malaria is a disease caused by parasites belonging to the apicomplexans family in the genus Plasmodium. It is transmitted by sixty of the two hundred species of female anopheline mosquitoes. They bite during the night hours from dusk to dawn.

Mechanical transmission could also occur during blood transfusion but is rare. The species responsible for human malaria are; *Plasmodium vivax*, *Plasmodium falciparum*, *Plasmodium ovale* and *Plasmodium malarie* (Suh *et al.,* 2004) Asexual multiplication of the malaria parasite is the prerequisite for the development of the clinical symptoms and disease in the human host (Woodrow *et al.,* 1999).

1.2.2 Life Cycle

The human malaria parasite requires both a human host and a vector (female anopheline mosquito) for transmission. The mosquito infects humans when taking a blood meal mainly by injecting the sporozoites into the subcutaneous tissue and less frequently into the blood stream. They selectively invade the hepatocytes in the liver cells before invading the red blood cells (Weatherall *et al*., 2002).

Once in the cells, the sporozoites develop into schizonts within two weeks. One schizont contains hundreds of merozoites and once it matures, it ruptures and releases the merozoites into the blood stream. The merozoites then invade the red blood cells and under go distinct developmental asexual stages, from the ring passing through trophozoite to schizont. These asexual stages feed on haem in the red blood cells and infect other hepatocytes making the extra-erythrocytic cycle (Phillips, 2001; Gazarini and Garcia, 2003).

The majority of the merozoites go through another asexual cycle in the red blood cells, to release merozoites that infect more new red blood cells. A small proportion of merozoites switch to sexual development (gametocytes). In some species a few merozoites switch into hyphozoites (dormant stage). Male and female gametocytes develop within the red blood cells and mature in four to ten days. While in the blood

circulation, they are taken up in a blood meal by a female anopheline mosquito (Phillips, 2001; Baker, 2004).

Once in the mosquito's stomach the gametocytes develop to gametes and are released from the red blood cells. Fertilization takes place forming an ookinete that burrows through the mosquito gut and forms an oocyst. Once the oocyst matures, it ruptures and releases the sporozoites that migrate to the salivary glands awaiting injection into the next human host during a subsequent blood meal (Niaid *et al*., 2002; Hastings, 2003).

Plasmodium parasites are haploid during the asexual phase and are capable of self-fertilization (a single infection can produce both male and female gametes). It is because of this haploid nature that the sexual phase occurs between identical genotypes and novel gene combinations are produced. Mating can occur between species in mixed infection (Out crossing). This results in recombination of genes both producing a new gene combination and breaking down the existing combination. Mixed infection is commonly found in areas of high transmission (Hastings, 2003).

1.2.3 Occurrence, Epidemiology & Economic impact

Malaria primarily occurs in the tropical and sub tropical areas, especially Asia, Africa and Central South America as shown in figure 1. It is estimated that 40 % of the world's population lives in malaria endemic area. It causes 300 million episodes of acute illness every year (Breman, 2001). The mortality levels are greatest in the sub-Saharan Africa where children below five years of age are most vulnerable resulting in 90 % of the deaths due to malaria (Breman, 2001).

The four species of human Plasmodia have different geographical distributions. *P. falciparum* is predominant in Africa. *P. vivax* is found mostly in Asia, Latin America and parts of Africa. *P. ovale* is found mostly in West Africa and the islands of the western Pacific and *P. malarie* is found scattered worldwide (http:// www CDC .gov/malaria).

The parasites cause diverse and sometimes fatal clinical conditions in human. In small proportions of infections, untrammeled parasite multiplication may lead to high parasite density resulting in dysfunction of vital organs. This may result in impaired consciousness, acidosis, severe anemia and death. *P. falciparum* accounts for the majority of instances of morbidity and mortality (White, 2004).

Malaria imposes substantial costs to both individuals and governments. This includes; costs of anti-malarials, expenses for traveling to health centers, lost days of work, absence from school, expenses for preventive measures and costs of public health interventions. These costs have immense effects on the economic and social burden of malaria in endemic countries and impede their economic growth (Suh *et al*., 2004; WHO, 2001). Statistics have shown that the annual economic growth rates over a period of 25 years in malaria endemic is 1.5% lower than in the other countries (Gallup and Sachs, 2001).

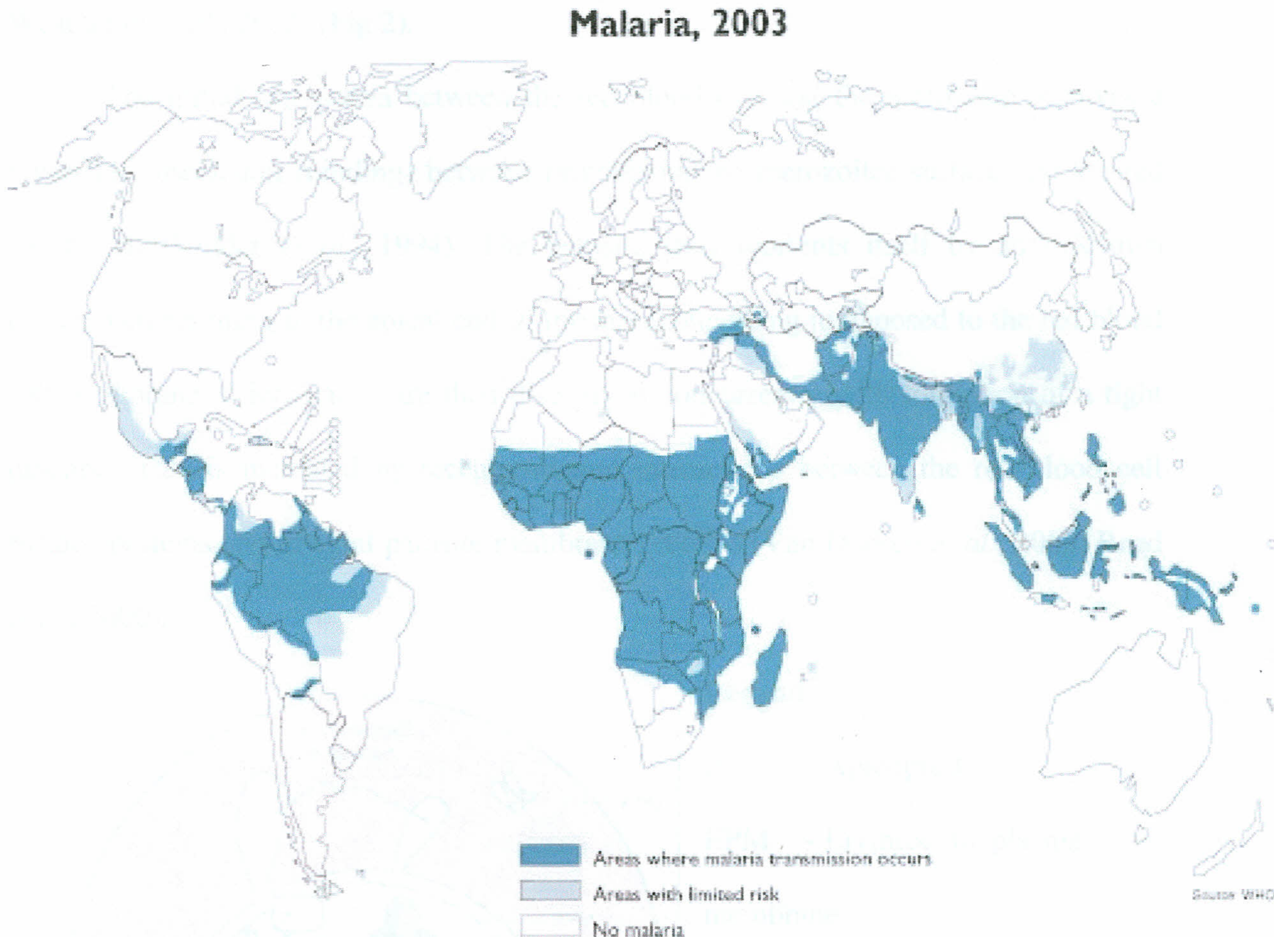


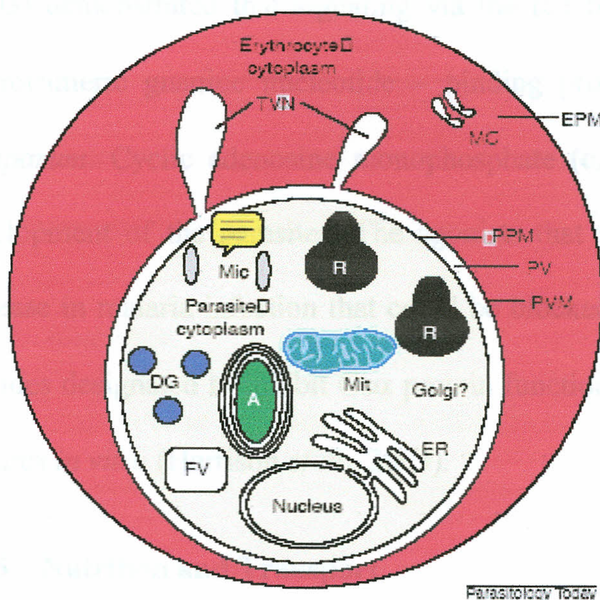
Figure 1.The global distribution of malaria (http://www CDC. gov / malaria)

1.2.4 Parasite - host cell interaction & invasion

Merozoites specifically enter the red blood cells using receptor-ligand interactions. This process is complicated and studies to understand it at molecular and cellular levels are on going (Gratzer and Dluzewki, 1993). The receptors involved in sporozoite invasion involve the thrombospondin domains on the circumsporozoite protein (CSP) and on thrombospondin-related adhesive protein (TRAP). These domains bind specifically to heparin sulfate proteoglycans on hepatocytes in the region in apposition to sinusoidal endothelium and kuppfer cells. The three organelles on the invasive (apical)

end of a parasite are rhoptries, micronemes and dense granules (Van Dooren *et al*., 2000; Weatherall *et al*., 2002) (Fig 2).

The initial interaction between the red blood cell and the merozoite involves a reversible interaction (binding) between proteins on the merozoites surface and the red blood cell (Holder *et al*., 1994). The parasite then reorients itself by an unknown mechanism resulting in the apical end of the merozoite being juxtaposed to the red blood cell membrane. Micronemes are then discharged concurrently with formation of a tight junction. This is mediated by receptor–ligand interactions between the red blood cell surface proteins and integral parasite membrane proteins (Van Dooren *et al.*, 2000; Reed *et al.*, 2000).



Legend:

A – Apicoplast

EPM – Erythrocytic plasma membrane

ER – Endoplasmic reticulum

FV - Food Vacuole

MC - Maurer's clefts

Mic - Micronemes

Mit – Mitochondrion

PPM – Parasite plasma membrane

PV –Parasitophorous vacuole

PVM–Parasitophorous vacuolar membrane

R - Rhoptries

TVN –Tubulovesicular network

Figure 2: A parasitized red blood cell showing the organelles of the malaria parasites

Red blood cell's membrane proteins are redistributed at the time of this junction formation to free the contact area from the proteins. An incipient parasitophorous vacuolar membrane (PVM), which is derived from both the parasite contents and the red blood cell membrane forms in the junction area. Rhoptries discharge their contents while the PVM expands as the parasite enters the red blood cell. Once the invasion is complete, the dense granules release their contents to PVM. The merozoites move through the ring shaped tight junction formed by the receptor-ligand complex and there after the PVM closes (Van Dooren *et al.,* 2000; Reed *et al*., 2000).

Several cell mediated signals and responses are involved in the invasion and developmental stages of the Plasmodium parasites. A study done by Harrison *et al.* (2003) demonstrated that signaling via the red blood cell β2- adrenergic receptor and heterotrimeric guanine nucleotide – binding protein (Gsα) regulated the entry of *P. falciparum*. Cyclic adenosine monophosphate (cAMP) plays an important role in the development of the parasites. The agonists that stimulate cAMP production led to an increase in malaria infection that could be blocked by specific receptor antagonists. The peptides designated to inhibit Gsα protein function reduced parasitemia in *P. falciparum* cultures *in vitro* (Harrison *et al*., 2003).

1.2.5 Nutrition and Transport

The parasite imports host cell hemoglobin by invagination of the parasite plasmalemma within the host cell cytoplasm at the micro-pore. Several other substrates are imported from extra-cellular milieu. They include; glucose, dicarboxylic acids, purines and specific amino acids. The potential mechanisms involved in the uptake are: parasite-induced permeability changes in the red blood cell plasmalemma, direct

connection between parasitophorous duct and the external milieu and involvement of tubovesicular membrane (TVM) network through directed transport across the red blood cell cytoplasm (Elford *et al.*, 1997).

The intra-erythrocytic development of the parasite imposes a major new demand for the supply of nutrients and waste disposal in the host cell. The asexual parasites are dependent on glucose from the host as a source of energy during multiplication. An increase in metabolic activity is associated with the enlargement of the parasite within the red blood cell. To satisfy the increased demands, the parasite causes profound alterations in the permeability of the host red blood cell membrane, increased production and export of lactic acid by the infected red blood cells (Woodrow *et al.*, 1999; Gero *et al.*, 2003). The possible entry and exit routes of the solutes in the intra-cellular parasite are via a metabolic window formed at a close apposition of the host red blood cell membrane, the parasitophorous vacuole membrane (PVM) and the parasite plasma membrane (PPM) via a membrane duct (Gero *et al.*, 2003).

1.2.6 Diagnosis

High rates of misdiagnosis of malaria have been reported. In sub-Saharan Africa, more than 80 % of individuals take anti-malarials to treat fevers without proper diagnosis, resulting in an increase in drug resistance (Barnish *et al.*, 2004). Reliable diagnosis cannot be made on the basis of signs and symptoms alone and hence presumptive diagnosis is used in conjunction with the definitive diagnosis.

Occasionally, severe disease can be present without detectable parasites in the peripheral circulation. If a patient is seriously ill and with clinical symptoms (suspicion) of malaria infection, then the empirical therapy could commence even with no detectable

parasites in the peripheral blood (Weatherall *et al.,* 2002). Rapid and accurate diagnosis is the key to effective disease management and one of the basic strategies for malarial control (WHO, 2000).

Direct microscopic examination of a stained blood film is the current standard method for definitive malaria diagnosis (Proux *et al*., 2001; Barnish *et al*., 2004). A well-prepared thick and thin blood films are stained by use of rowmanknowsky stains preferably the giemsa stain. Advantages of this method include; differentiation between species, quantification of the parasite density and ability to distinguish the clinically important stages in the parasites. The proper diagnosis is critical for proper disease management and treatment (Bloland, 2001).

There is a modified method of light microscopy known as quantitative buffy coat method (QBC ™, Becton-Dickinson). A fluorescent dye that has a high affinity to the nucleus of the malaria parasites is coated at the end of a special capillary tube. Upon exposure to UV light of 390 nm wavelength, the fluorescent dye rises to a higher energy level. The nucleus then takes up the dye and an electron is lost resulting in the fluorescence of the nucleus. This method uses micro-hematocrit tubes pre-coated with fluorescent acridine orange stain to highlight the malaria parasites. Upon centrifugation, the parasites are concentrated at a predictable location. This method is quicker and has been found to be more sensitive especially in cases of low parasitemia than conventional microscopy. It has more than 95 % sensitivity and specificity (Bloland, 2001; Moody, 2002).

Antigen detection tests using rapid immuno-chromatographic techniques are used as a diagnostic tool. These tests require no special equipment but are disadvantaged in that, they can't quantify the parasite density (Bloland, 2001; Barnish *et al*., 2004). Malaria antigens suitable for use as targets are Plasmodium Lactate Dehydrogenase (pLDH) and Histidine Rich Protein –2 (HRP-2).

HRP-2 is a water-soluble protein and is only produced in abundance by asexual stages and young gametocytes of *P. falciparum*. pLDH is an enzyme found in the glycolytic pathway of all Plasmodium species and is produced by sexual and asexual stages. Aldolase enzyme can be used as an indicator of malaria infection (Moody, 2002). Serology is used whereby the antibodies in serum are detected by use of specific serology markers. This technique has no role in the diagnosis of acute malaria (Suh *et al*., 2004).

Detection of specific nucleic acid sequences using Polymerase Chain Reaction (PCR) technique has become a more frequent tool in diagnosis. It is able to differentiate between species in a mixed infection and may be used where the light microscopy is inconclusive. It may be used for diagnosis only where a large number of samples are involved because of the high cost of the reagents involved (Bloland, 2001). It is disadvantaged in that it tests positive 144 hours after a successful treatment (false positive) (Moody, 2002).

1.2.7 Chemotherapy

1.2.7.1 Chemotherapeutic agents

4-aminoquinolines (chloroquine and amodiaquine) and quinolinemethanols (quinine and mefloquine) have been the main strays of malaria chemotherapy for the past four decades. The success of these anti-malarials has been due to clinical efficacy, limited

host toxicity, ease of use and cost effective (low cost drugs). The efficacy has decreased in the recent years due to development and spread of parasite resistance (Giancarlo *et al.*, 2003).

4-aminoquinolines are active only against the parasites in the intra-erythrocytic cycle during which the parasite is actively degrading hemoglobin (Foley and Leann, 1998). They inhibit the parasite–specific process of hemoglobin degradation. The activity of chloroquine is dependent on the external pH and proton pump inhibitors. The proton pump inhibitors antagonize its mechanism of action (Macreadie *et al*., 2000).

The primary mode of action of chloroquine utilizes heme as the target by inhibiting its polymerization to hemozoin. Heme is toxic to the parasites hence the inhibition of hemozoin formation leads to the poisoning of the parasite. Heme is also toxic to the cells by a free radical dependent mechanism. When it is released from its protein environment, oxidation of the iron from the Fe^{2+} to the Fe^{3+} state occurs. This results in the production of reactive oxygen species (super oxide anion, hydrogen peroxide and hydroxyl radicals), which cause oxidative stress in the cell (Foley and Leann, 1998; Giancarlo *et al*., 2003).

The activity of chloroquine mainly depends on the drug accumulation in the parasite. The major mechanisms postulated to account for chloroquine accumulation are; trapping of charged molecules in the acidic (pH 5.3) food vacuole, active uptake of chloroquine by a specific transporter (both are ATP dependent) and binding of chloroquine to an intra-cellular receptor (ferriprotoporphyrin IX), which is ATP-independent (Macreadie *et al.,* 2000).

Quinolinemethanols target the food vacuole of the parasites. Mefloquine causes morphological changes in the food vacuole of the parasite and appears to cause degranulation of hemozoin. Quinine inhibits heme polymerization and its catalytic activity. 8-aminoquinolones (primaquine) are active on gametocytes and hyphozoite stages where they interfere with the activity of the mitochondrion. Atovaquone also interferes with the activity in mitochondrion (Egan *et al*., 1994; Foley and Leann, 1998).

Doxycycline is a slow acting drug which is known to target plastid 16S rRNA hence blocking the apicoplast translation system. It may also inhibit the mitochondrial protein synthesis (Ralph *et al*., 2001). Artemisinin and its derivatives interact with intra parasitic heme or iron. It then activates artemisinin in the parasite resulting in formation of free radicals that damage the specific target organelles (membrane via lipid peroxidation) (Meshnick, 2002).

The anti-malarials which were previously effective for chemotherapy have been incapacitated due the development of resistant parasites. The major avenues to combat the problem are through: determination of the genome sequence of *P. falciparum* that offers a multitude of potential drug targets, anti-malarial drug discovery and development (Fidock *et al*., 2004), identification of enzymes that may serve as potential targets for destroying the parasites (Waters and Geyer, 2003), drug combinations and natural products (Rosenthal, 2003).

Anti-malarial drug discovery has been made possible by *in vitro* techniques, which evaluate the potential anti-malarial activities of new and potentially novel drugs (Milhous *et al*., 1989). Some of the efforts that are currently on going in search for new effective anti-malarials are; optimization of therapy with available drugs including the

use of combination therapy, the development of analogs of existing agents, the discovery of natural products, the use of compounds that were originally developed against other diseases, the evaluation of drug resistance reversers and the evaluation of new chemotherapeutic targets (Rosenthal, 2003).

1.2.7.2 Potential targets for chemotherapy

Invasion of the red blood cells by the parasites results in an increased demand of nutrients in the host cell through various pathways. Regardless of the route of entry or exit of the nutrients (solutes) to or from the host cell, there is induction of transport pathways that are different from those in other mammalian cells (Gero *et al.*, 2003).

There are two different changes in transport of solutes upon infection, increased flux via transport pathways similar to the endogenous host cell transporters and new induced permeation pathways with functional characteristics different from those in the uninfected cell. It is indicated that the transporter in the latter pathway may be a pore and not a conventional carrier (Gero *et al.*, 2003).

Enzymes of the *de novo* pyrimidine biosynthetic pathway have been the major focus for anti-metabolic therapy. Other pathways that are of interest include the fatty acid biosynthetic pathway, calcium homeostasis mechanism and polyamine biosynthesis and its regulation. Irrespective of the route, the induction of transport pathways in the infected red blood cells offers potential opportunities for anti-malarial chemotherapy. The potential anti-malarials could be toxic compounds that are selectively transported into the parasite-infected host cell by the induced transport systems or the inhibitors of the parasite induced transporters (Gero *et al.*, 2003).

Magnesium is an essential cation for several major metabolic cellular processes. Its high uptake rate in the infected cells with *P. falciparum* was mediated through Furosemide – sensitive New Permeation Pathways (NPP) induced by the parasite or through ATP depletion - induced calcium permeability pathway (Macreadie *et al*., 2000). Several compounds have been shown to inhibit this pathway, for example, phlorizin, sulfonylureas, chalcones and arlaminobenzoates (Ralph *et al*., 2001). Unfortunately the activity of these compounds is too low to be pharmacologically relevant. L-adenine analogs penetrate poorly into uninfected red blood cells but enter into an infected cell through NPP and parasite plasma membrane (PPM). The parasite enzymes render these analogs toxic to the parasites (Macreadie e*t al*., 2000).

The isolation and characterization of a novel parasite–encoded hexose transporter PfHT 1 that is associated with the PPM explains transport of glucose in different stages of parasite during an infection. It is a potential drug target because the anti-malarial used in the management of severe malaria infection take a long time to achieve maximum inhibition of glucose utilization. PfHT 1 inhibitors may be rapidly parasitocidal (Woodrow *et al*., 1999). Once the PPM transporters are blocked, there is reduction of ATP levels and loss of pH homeostasis resulting to death.

The unique properties of NPP can be used to target the red blood cells by drugs / compounds that otherwise cannot penetrate the host cells. Nucleoside analogs use NPP and PPM transporter systems to penetrate the red blood cells (Biagini *et al.,* 2005). Phospholipid (PL) metabolism is absent in the mature red blood cells but is rampant in the infected red blood cells. It (lipid) is mostly derived via de novo pathway from

phosphatidyl choline (PC). Choline transporter is essential in the lipid metabolism and hence posses as a potential anti-malarial target (Biagini *et al.*, 2005).

The parasite's digestive food vacuole has two proton-pumping mechanisms: Bafilomycin sensitive V-type and Na F–sensitive H^+ - pyrophosphatase showing a partial dependence on Potassium ions (K^+). There is evidence showing that calcium storage by the food vacuole is linked directly to the parasite cell signaling and asexual development. The requirement for tightly regulated calcium ions (Ca^{+2}) movement and storage in this organelle could be used as a target for the mode of action of a new chemotherapeutic agent (Biagini *et al.*, 2003).

1.2.7.3 Drug combinations

This is a rational approach to combat drug resistance, which avoids the long wait of drug development. The use of single drugs that are easily prone to resistance limits therapeutic choice hence, the emergence of combination therapy. This is true in cancer, HIV/AIDS, leprosy, tuberculosis and now in malaria therapy (Bloland, 2001). Combination therapy includes use of newer agents for example, artemisinin derivatives, atovaquone and older agents for example, amodiaquine, sulfadoxine, pyrimethamine, chlorproguanil, and dapsone. Slow acting anti-malarials such as antibiotics may work well in combination regimens. A good combination should improve anti-malarial efficacy and slow the progression of parasite resistance to new agents (Bloland, 2001; Rosenthal, 2003).

These combinations could result in pharmacological interaction between the components hence altered disposition or toxicity therefore a need to perform

pharmacokinetics interaction studies (White, 1999). The components used in a combination should have independent modes of action and different biochemical targets in the parasites (Bloland, 2001). This enhances their respective efficacies and therapeutic half-life (Taylor *et al.,* 2001). The basic tenet of combination therapy is that the probability of resistance developing simultaneously in two chemotherapeutic agents with independent mechanisms of action is extremely low (1 in 10^{12} treatments). This frequency is the product of the probabilities of acquisition of a resistant mutation to each drug multiplied by the number of parasites in a typical infection (White, 1998). The anti-malarial activity of the combined drug may either be synergistic, antagonistic or additive.

WHO has classified anti-malarial combination therapy into two, non-artemisinin based and artemisinin based combinations. The advantages of the artemisinin-based combinations are the unique properties and its mode of action. These include; limited reported adverse clinical effects, lack of parasite resistance and its efficacy against multi drug–resistant *P. falciparum* (Bloland, 2001).

Sulfadoxine-pyrimethamine (SP) had been a widely used combination that replaced chloroquine as the first line treatment for uncomplicated *P. falciparum* malaria. Its resistance has been reported in several countries and it is no longer used as a drug of choice (Taylor *et al*., 2001). Studies on chloroquine-SP combinations in Gambia and Papua New Guinea have shown a higher efficacy than that of SP alone. This combination was used as a first line treatment in Peninsular Malaysia and Papua New Guinea in 1997 and 2002, respectively. It was shown to be non-effective on chloroquine resistant strains (Bloland, 2001). A study done on chloroquine-doxycycline combination in Indonesia

demonstrated a 90 % cure rates in *P. falciparum* infections. It had lower curative rates in *P. vivax* infections and the resistant strains of *P. falciparum* (Taylor *et al*., 2001).

Amodiaquine-SP combination was demonstrated to be of no significance. This is because it had an efficacy of 95 %, which was similar to that of amodiaquine as a mono-therapy (WHO, 2001). Mefloquine-SP (MSP) had an additive activity and was shown to have no pharmacokinetic interaction between the components. Its use as a first line treatment in Thailand for uncomplicated malaria resulted in rapid development of resistance (Bloland, 2001).

Atovaquone is believed to be a potent inhibitor of electron transport. When used as a single agent, resistance developed rapidly, whereas in combination with doxycycline or proguanil, it exhibited remarkable potentiation of the drug effects (Canfield *et al*., 1995; Hyde, 2005). Atovaquone–proguanil (malarone) was found to have a synergistic effect and was highly efficacious against *P. falciparum* including the chloroquine resistant strains (WHO, 2001; Wichman *et al*., 2004).

Azithromycin, a poor anti-malarial as a single agent, demonstrated synergistic activity when used in combination with chloroquine on chloroquine resistant *P. falciparum* strains and demonstrated additivity on chloroquine sensitive strains. It also demonstrated additivity to synergistic activities when used in combination with quinine, tafenoquine and primaquine (Ohrt *et al*., 2002). Clinical trials done in Kenya demonstrated that a new analog of erythromycin, azithromycin was effective against *P. falciparum* in areas of intense malaria transmission (Andersen *et al*., 1998).

Artesunate in combination with mefloquine and quinine revealed synergistic activity on chloroquine sensitive and chloroquine resistant strains. It revealed additivity

when used in combination with doxycycline and antagonism in combination with chloroquine on both W2 and D6 (Fivelman *et al.*, 1999). Artemether-lumefantrine (Coartem) is the most viable artemisinin combination treatment available. It is safe, has high efficacy and tolerance profile (WHO, 2001).

1.2.8 Resistance

The parasites develop resistance to anti-malarials when the required concentration of an anti-malarial spontaneously eliminates the more sensitive parasites with exception of the mutant parasites. The selection of the mutants most likely occurs when; a large number of parasites encounter sub-maximal concentration of the anti-malarial drug in the blood, the primary treatment is inadequate or when a newly acquired infection encounters residue concentration of the anti-malarial from treatment of a previous infection (Simpson *et al.,* 2000).

In the later case the chance of resistance selection is higher for anti-malarial drugs with long elimination half-lives. This is because the drugs remain in the blood at sub-therapeutic concentration for a longer duration (Watkins and Mosobo, 1993). In drug resistant infections, parasites could remain in peripheral blood at very low concentrations that are undetectable by microscopy and recur at a later time or increase following the administration of anti-malarials (White, 2004).

The emergence of resistance especially in *P. falciparum* is the major contributor to the global resurgence of malaria in the last three decades. It results from lack of inhibition of parasite multiplication at any given level of anti-malarial plasma concentration (White, 2004). Malaria parasites carrying genes conferring resistance to

anti-malarials have higher rates of transmission in the drug treated host (Hallet *et al.*, 2004).

The two basic mechanisms of resistance are; altered binding affinity and altered drug transport. Chloroquine resistance results from a reduced drug accumulation in the parasite's food vacuole (Su *et al.*, 1997; White, 1999). Chloroquine resistant strains of *P. falciparum* are known to rid themselves of the drug forty to fifty times faster than the chloroquine sensitive strains. Wellems and colleagues investigated the mechanism of this efflux by determining the genetics of chloroquine resistance. This was made possible by the fact that the efflux appears to be controlled by a single loci hence the use of restriction fragment length polymorphism (RFLP) analysis to map this locus to a specific chromosome region (Wellems *et al.*, 1991).

The rapid efflux in the chloroquine resistant phenotype is governed by a single genetic locus on chromosome 7 (Wellems *et al.*, 1991). Chloroquine resistant trait in *P. falciparum* has been mapped to a 36 kb segment of chromosome 7 which harbors cg2 gene with complex polymorphism (Su *et al.,* 1997). Chloroquine transport takes place at the parasite's periphery where the CG2 protein is located and in association with hemozoin in the food vacuole (Su *et al.*, 1997).

The resistant type of P-glycoprotein has been linked to chloroquine resistance. There is a genetic linkage between loci on chromosome 5, 7, and 13 and the chloroquine resistant phenotype. The 2 genes identified to confer resistance are pfmdr 1 and pfCRT on chromosome 5 and 7. PfCRT is the protein that functions as a transporter in parasite's digestive vacuole membrane. The gene on chromosome 13 has not been identified (Macreadie *et al.*, 2000; Wellems and Plowe, 2001). Pfmdr 1 gene mutations or

amplification are insufficient to cause chloroquine resistance independently because Pgh – expressing cell, can effectively transport the drug into the cell (Wellems *et al.*, 1990; Van Es *et al.*, 1994; Volkman *et al.*, 1995).

Increased gene copy number and expression of Pfmdr has been correlated with resistance to mefloquine and halofantrine in *P. falciparum* isolates from Thailand. The lower mefloquine dose provides a greater opportunity of de novo selection of genetic mutants, especially in those that contain increased copies of Pfmdr 1 gene. Its low elimination rate increases the chances of developing resistance than if a lower dose was administered (Wilson *et al.*, 1993; Price *et al.,* 1999).

A complex series of polymorphism in cg2 gene correlate with chloroquine resistance in *P. falciparum* parasites from South East Asia although there was no correlation in the parasites from South America (Watkins and Mosobo, 1993). Chloroquine resistance spread inexorably from South East Asia and South America to almost all the malaria endemic regions. This resulted in the increase of sulfadoxine-pyrimethamine (SP) use as a first line treatment for *P. falciparum* malaria. This combination led to production of resistant populations of *P. falciparum* and could no longer be used as the drug of choice.

The resistance of SP is associated with point or multiple mutations in DHFR for pyrimethamine and DHPS for sulfadoxine (Macreadie *et al.*, 2000). Mefloquine resistance correlates well with resistance to halofantrine and quinine although it has inverse relations to chloroquine (White, 1999).

Mutations in gene encoding cytochrome b were detected in isolates of Atovaquone resistant *P. falciparum*. Some mutations reduced atovaquone susceptibility

by 9354 fold and were located in a putative drug-binding site. The first cases of malarone resistance have been reported and are associated with mutations at codon 268 of the parasite's cychrome b gene in Central Africa (Wichman *et al.*, 2004). There are other reported cases of resistance to malarone not associated with codon 268 (Farnert *et al.*, 2003).

1.2.9 Control

The goal of malaria control in malaria endemic countries is to reduce as much as possible the health impact of malaria on the human population. This is made possible by use of the available resources and taking into account other health priorities. Eradication is more desirable but is not a realistic goal at present. Efforts to eradicate malaria have been unsuccessful in many regions of the world in the past. Currently, efforts to control malaria focus on reducing morbidity and mortality attributed to the disease (Suh *et al.*, 2004).

Malaria control is carried out through several interventions that are often combined, namely; chemotherapy and Vector control (Phillips, 2001; Shift, 2002). Malaria vaccine trials are on going, however, they have up to date failed (White, 2004).

Shortcomings of malaria control include: resistance to anti-malarial drugs and insecticides, the decay of public health infrastructure, population movements, political unrest and environmental changes which contribute to the spread of the parasites (Greenwood and Mutabingwa, 2002). The insecticide resistance decreases the efficacy of interventions that rely on vector control for example, treated bed nets and indoors spraying. The result of this is inability to conduct the recommended interventions

effectively. The poor countries are unable to conduct the recommended interventions due to inadequate health infrastructures (Phillips, 2001).

1.2.10 Cell signals

1.2.10.1 G-Proteins

Heterotrimeric guanine nucleotide-binding regulatory proteins (G proteins) constitute a well-characterized class of transduction proteins in mammalian cells. They are found at the cytoplasmic face of the cellular membrane where they can couple with a variety of trans-membrane proteins to transduce extra-cellular signals. This is initiated by, hormones, neurotransmitters and autocrine and paracrine factors (Harrison *et al.,* 2003). The activation of G proteins involves guanine nucleotide exchange factors that promote dissociation of guanine diphosphate (GDP) from the inactive G protein and replacement with guanine triphosphate (GTP) (Harrison *et al.,* 2003). The effector proteins of the G βα complex include adenylyl cyclase, phospholipase and ion channels (Ford *et al*., 1998). Although G proteins have been intensively studied, their function in mature red blood cell is poorly understood (Harrison *et al.,* 2003).

1.2.10.2 Adenylyl cyclase

This is a family of membrane bound enzymes that are involved in wide range of cellular processes in the cell. These activities include regulation of ion channels in wide range of cells and catalyze the formation of 3′ 5′ – cAMP from ATP (Desaubry *et al.,* 1996; Hurley, 1999). The regulation of cAMP involves several signaling processes evolving between the receptor (example, G-proteins) activation and communication to the effector proteins (Example, adenylyl cyclase). G-protein - dependent adenylyl cyclase

and adenylyl cyclase (AC) of higher eukaryotes conform to the same basic structure consisting of two catalytic domains C1 and C2. The GTP – bound G protein α subunit ($G_s\alpha$) potently and physiologically activates all the mammalian AC. This activation is synergistic and not competitive (Baker and Kelly, 2004 b; Ford *et al.,* 1998).

There are ten known isoforms in mammalian cells that are regulated by numerous hormones and neurotransmitters via surface receptor linked by stimulatory G_s and inhibitory G_i. Activation occurs indirectly when a ligand binds to a G protein coupled receptor, which in turn interacts with heterotrimeric G-proteins resulting in dissociation of this complex heterotrimeric G-proteins into free G_α and $G_{\beta\gamma}$ subunits (Baker and Kelly, 2004 a; Ford *et al.,* 1998). There is exchange of GDP –bound G_α for GTP on the G_α subunit. The free heterotrimeric G-protein subunit (G_α) in turn binds to AC hence stimulating the synthesis of cAMP (Baker and Kelly, 2004 a; Ford *et al.,* 1998).

Adenylyl cyclases are divided into six classes whereby class III cyclases (the universal class) are found in both the Eukaryotes and prokaryotes (Baker and Kelly, 2004 b). Both G- proteins and plant diterpene (Forskolin) regulate mammalian trans-membrane AC. Forskolin is a powerful activator and it activates AC directly (Hurley, 1999; Baker, 2004).

A mammalian bound AC contains two hydrophobic spans composed of six trans-membrane helices and three large cytoplasmic domains. It is clearly demonstrated that the two cytoplasmic domains (C1 and C2) form the catalytic core. It has conserved residues that include; asparagine or arginine for stabilizing the transition state of the enzyme, lysine or glutamate that determines adenine or guanine binding respectively.

These residues are however less conserved among lower eukaryotes and prokaryotes (Baker and Kelly, 2004 b).

Adenylyl cyclases are potently and directly inhibited by analogs of adenosine via P- site domain. P-site inhibitors are a class of nucleosides inhibitors of AC that contains purine ring. They bind to the active site of AC through conserved residues (Hurley, 1999). All mammalian AC tested have been shown to be susceptible to P-site mediated inhibition except for those from the sperm cell. Although ligands (P-site) act directly on the enzyme, hormone stimulation is more sensitive (Desaubry *et al*., 1996; Johnson *et al.,* 1997). Type 1 AC is inhibited by P-site ligands and via cell surface receptors or G-proteins. It was demonstrated that the inhibitory potencies were dependent on the isozyme type (Johnson *et al.,* 1997).

The most potent AC inhibitor isozyme lack a 2′- hydroxyl and are phosphorylated at 3′ position. The inhibitors bind to the active site primarily through conserved residues. They are non competitive in the forward reaction but compete with cAMP in the reverse reaction (Hurley, 1999). The inhibitory potency of some isozyme from the lowest is as follows; 9-tetrahydrofuryl adenine, 9-cyclopentyladenine, β-adenosine and 2′ 5′ dideoxyadenosine (Johnson *et al.,* 1997).

In Plasmodia, there are two ACs. One, PfACα, which has a membrane associated N-terminal domain is detectable in gametocytes and its bi functional structure implies that electrical fluctuations resulting from the environment ion concentrations could be coupled to cAMP synthesis. Second one is PfACβ, which is closely related to the soluble AC (Baker and Kelly, 2004 b).

The predicted structure of this enzyme is unique to the apicomplexans, paramecium and tetrahymena. The C-terminal catalytic domain has all the amino acids residues necessary for substrate and magnesium co-factor binding. The predicted purine binding residues are consistent with those found in other AC. Baker demonstrated that there were relatively low levels of PfACα mRNA expression in asexual blood stages, gametocytes and sporozoites. This study revealed a higher level of PfACβ mRNA expression in the asexual blood stages reaching the peak at late schizonts (Baker, 2004).

1.2.10.3 Cyclic Adenosine Monophosphate

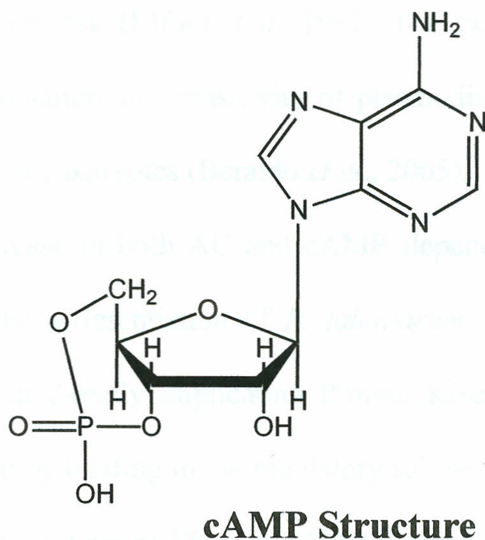


Figure 3: The structure of cAMP

The discovery of cAMP and the molecular dissection of its role in hormone action led the way for an era of biological research. cAMP is a heat stable factor that enables mammalian cells to respond to hormones. Cyclic nucleotides, cAMP and cGMP are ubiquitous signaling molecules synthesized by AC and GC respectively. They are second

messengers of intracellular events initiated by activation of many types of hormone and neurotransmitter receptors (Francis and Corbin, 1999; Simonds, 1999; Cann, 2004).

The second messengers mediate a myriad of cellular functions in the entire animal kingdom. This ranges from simple responses to environmental changes in lower animals and nerve cell communications in mammals. The activation of the α-subunit of the G protein stimulates the AC that catalyzes the conversion of cytoplasmic ATP to cAMP (Francis and Corbin, 1999; Simonds, 1999; Cann, 2004).

Intraerythrocytic development of plasmodia parasites depends on a series of complex signaling pathways in which the second messengers; for example, cAMP and calcium play a key role (Elford *et al.,* 1997). The molecular machinery controlling cAMP synthesis, degradation and sensitivity of plasmodia has been described to be similar to that of the higher eukaryotes (Beraldo *et al*., 2005).

An increase in both AC and cAMP–dependent Protein Kinase (PKA) activities accompanies the differentiation of *P. falciparum.* The role of cAMP has mostly been reported without directly implicating Protein Kinase A (PKA). In several instances it exerts its action by binding to the regulatory subunit complexed to the catalytic subunit in an inactive holoenzyme of PKA or cAMP-dependent Protein Kinase. This results in the release of the active catalytic subunit (PKA-C). PKA activity can be regulated through binding of its natural inhibitor (PKI). Structural considerations suggest that it might be possible to inhibit the parasite PKA rather than the red blood cell enzyme (Endogenous PKA). Such compounds would be potential anti-malarials (Syin *et al.,* 2001).

cAMP has been implicated in regulation of sexual differentiation in *P. falciparum.* It was demonstrated to have cytotoxic effects to the parasites under certain

conditions. Inselburg demonstrated this and showed that cAMP exhibits a stage specific inhibitory effect on asexual development and that it stimulates gametogenesis in some of the *P. falciparum* cell lines (Inselburg, 1983).

The morphological changes found in an infected cell are abnormalities in membrane proteins, degradation of certain proteins and appearance of other possibly new polypeptides. Some of these changes reflect damage to membrane but some may be metabolic windows to facilitate the provision of intra-cellular parasite with essential nutrients (Hertelendy *et al.*, 1979).

Cellular levels of cAMP in mature murine red blood cells are relatively low compared to other cell types. However, there is a drastic rise of cAMP in the red cells upon malaria infection. This change is confined in the red blood cell, as there is no change in cAMP concentrations in the circulating plasma. The importance of cAMP increase has been demonstrated in both the parasite and its host. It has been shown to participate in the growth and development of cells. The fundamental regulatory processes make it likely that the alteration in cAMP metabolism is of physiological importance and may be useful in designing new anti-malarials (Sheppard *et al.*, 1981).

The cAMP response element (CRE) consensus sequence is involved in the transcription of a wide range of genes. A 24-mer single stranded cAMP response decoy oligonucleotide (CDO) competes with these sequences for binding transcription factors resulting in transcription inhibition. CDO as a single agent was demonstrated to have no effect on the cell proliferation to the tumor cells used. When used in combination with conventional chemotherapeutic drugs it showed synergistic anti-cancer effects in some of the colorectal cancer cell lines (Liu *et al.*, 2004).

1.3 Statement of Problem

The evolution of multi-drug resistance raises fear that emergence of resistance to anti-malarials may proceed faster than the development of new and effective drugs, therefore, the need to get ways of optimizing the efficacy of the available anti-malarials.

Malaria parasites have intracellular signaling pathways through phosphoinositide, cAMP and calcium-dependent pathways (Weatherall *et al.*, 2002). Although the red blood cells mechanisms involved in malarial infections are poorly understood, it was demonstrated that signaling via red blood cell heterotrimeric guanine nucleotide – binding protein regulated the entry of human malaria parasite (Harrison *et al.*, 2003). The effect of cAMP on malaria chemotherapy however, has not been established yet.

1.4 Research Questions

i) Do adenylyl cyclase inhibitors have any effect on the efficacy of anti-malarials?

ii) Do adenylyl cyclase activators have any effect on the efficacy of anti-malarials?

1.5 Hypothesis

The modulators of cAMP have an effect on the efficacy of anti-malarials.

1.6 Objectives

The general objective of the study was to determine the effect of cAMP modulators on the efficacy of various anti-malarials.

1.6.1 Specific Objectives

i) To determine the inhibitory concentration at 50 % (IC_{50}) of selected cAMP modulators.

ii) To determine the effect of adenylyl cyclase inhibition on the efficacy of chloroquine, mefloquine, quinine, doxycycline and amodiaquine on chloroquine resistant (W2) and susceptible (D6) strains of *P. falciparum*.

ii) To determine the effect of adenylyl cyclase activation on the efficacy of chloroquine, mefloquine, quinine, doxycycline and amodiaquine on chloroquine resistant (W2) and susceptible (D6) strains of *P. falciparum*.

CHAPTER TWO

2. MATERIALS AND METHODS

2.1 Parasite Clones

Reliable methods of direct visualization and single cell micromanipulation have enabled selection of two *Plasmodium falciparum* clones for screening candidate drugs by Walter Reed Army Institute of Research (WRAIR) and World Health Organization (WHO) (Milhous *et al.*, 1989).

The two *P. falciparum* clones are W2 and D6. The W2, *P. falciparum* clone, which was obtained from CDC Indochina, is resistant to chloroquine, pyrimethamine, sulphadoxine and quinine but susceptible to mefloquine. The D6 clone, which was obtained from CDC Sierra Leone, is resistant to mefloquine but susceptible to the other anti-malarials. These isolates were preserved in aliquots of 0.5 ml to 1.0 ml of parasitized cells in 6 % dimethylsulfoxide (DMSO) and cryopreserved in liquid nitrogen (-196 ºC).

2.2 Anti-malarials and cAMP modulators

The anti-malarials used in this study were chloroquine, mefloquine, quinine, doxycycline and amodiaquine. Five milligrams of each drug was weighed. Chloroquine was dissolved in 1.5 ml distilled water then in 3.5 ml absolute ethanol. Mefloquine and quinine were dissolved in 70 % ethanol while doxycycline and amodiaquine were dissolved in 100 % dimethylsulfoxide (DMSO) to obtain the stock concentration of 1 mg/ml. The stock concentration were then diluted further using plain media (RPMI with HEPES + $NaHCO_3$) to give a working concentration of 10,000 ng/ml for chloroquine, 2,500 ng/ml for mefloquine, and 20,000 ng/ml for quinine, 500,000 ng/ml for

doxycycline and 1,000ng/ml for amodiaquine. The working dilution contained less than 0.02 % DMSO or ethanol, which would not affect the parasites. These anti-malarials were obtained from Walter Reed Army Institute of Research (WRAIR)- USA and stored at room temperature in a desicator.

The cAMP modulators used were AC activator (forskolin), PKA activator (Sp-adenosine 3′ 5′ cyclic monophosphorothiate triethylammonium salt (A 166) and AC inhibitors; 9-cyclopentyladenine (9-cyclo) and 2′ 5′ dideoxyadenosine 3′ monophosphate (D0689). They were purchased from sigma–Aldrich Company and stored at -20° C. Five milligrams of each compound was weighed. Forskolin and D0689 were dissolved in 5ml of DMSO while A166 and 9- cyclo were dissolved in sterile double distilled water to obtain a stock concentration of 1 mg/ml. The stock concentrations were further diluted in stock media to give a working concentration of 500,000 ng/ml.

The stock concentrations were stored at – 80 °C for not more than three months whereas the working concentrations were prepared prior use. The working concentrations were divided by a factor 10 to obtain the final working concentration.

2.3 Experimental design

2.3.1 Malaria Cultures

The cultures were initiated by thawing the frozen samples (W2 and D6) at 37 °C for 10 minutes. They were transferred into 15 ml centrifuge tubes and washed twice (centrifuged at 2500 rpm for 5 minutes in 3.5 % sodium chloride, then in 10 % Complete Media with Serum). The cultures were maintained in 25 cm^3 culture flasks using 10 % Complete Media with Serum (RPMI 1640 + 7.5 % $NaHCO_3$ + 10 % Pooled heat

inactivated Serum), Blood group O positive cells at a hematocrit of 6 %, gassed using gas mixture (91 % N_2, 6 % O_2 and 3 % CO_2) and incubated at 37 °C.

The media was changed every day by aspirating the old media and adding fresh media (Trager and Jensen, 1976). Thin blood films were prepared thrice a week to monitor the development of the parasites.

2.3.2 *In Vitro* Drug Testing: Radioisotope Technique

A well-established technique for malaria drug sensitivity testing was used (Desjardins *et al.,* 1979). The drugs were tested on cultures upon attaining the parasitemia of 3 % to 5 % with at least 70 % of the parasites at ring stage. The anti-malarials and the cAMP modulators were first tested as single agents to determine the Inhibitory Concentrations that inhibited growth by 50 % (IC_{50}). The anti-malarials were then combined with the cAMP modulators in sterile tubes at the following dilutions, 1:1, 1:3, 3:1, 1:4, 4:1, and 1:5. An automated diluter was programmed to perform a two fold serial dilution for a total of eleven concentrations in a ninety-six well flat bottom micro titer plate (the template plate). The first two rows contained each drug alone and the other 6 rows contained the drugs in combination at different ratios. The twelfth column of the micro titer plate was untreated hence had the diluent only (control wells).

The automated diluter was programmed to transfer twenty five microlitres of the drug from the template plate into the wells of the working micro titer plate (coating). Two hundred microlitres of 1 % suspension of parasite-infected red blood cells was then added to the entire plate with exception of wells A12, B12 and C12 (they served as negative control wells). 1 % suspension of uninfected red blood cells in complete medium was added to the negative control wells.

Plate 1: A drug screening assay 96- well plate template

1	2	3	4	5	6	7	8	9	10	11	12
CDIMT A1	CDIMT A2	CDIMT A3	CDIMT A4	CDIMT A5	CDIMT A6	CDIMT A7	CDIMT A8	CDIMT A9	CDIMT A10	CDIMT A11	CIM A12
CDIMT B1	CDIMT B2	CDIMT B3	CDIMT B4	CDIMT B5	CDIMT B6	CDIMT B7	CDIMT B8	CDIMT B9	CDIMT B10	CDIMT B11	CIM B12
CDIMT C1	CDIMT C2	CDIMT C3	CDIMT C4	CDIMT C5	CDIMT C6	CDIMT C7	CDIMT C8	CDIMT C9	CDIMT C10	CDIMT C11	CIM C12
CDIMT D1	CDIMT D2	CDIMT D3	CDIMT D4	CDIMT D5	CDIMT D6	CDIMT D7	CDIMT D8	CDIMT D9	CDIMT D10	CDIMT D11	CIMT D12
CDIMT E1	CDIMT E2	CDIMT E3	CDIMT E4	CDIMT E5	CDIMT E6	CDIMT E7	CDIMT E8	CDIMT E9	CDIMT E10	CDIMT E11	CIMT E12
CDIMT F1	CDIMT F2	CDIMT F3	CDIMT F4	CDIMT F5	CDIMT F6	CDIMT F7	CDIMT F8	CDIMT F9	CDIMT F10	CDIMT F11	CIMT F12
CDIMT G1	CDIMT G2	CDIMT G3	CDIMT G4	CDIMT G5	CDIMT G6	CDIMT G7	CDIMT G8	CDIMT G9	CDIMT G10	CDIMT G11	CIMT G12
CDIMT H1	CDIMT H2	CDIMT H3	CDIMT H4	CDIMT H5	CDIMT H6	CDIMT H7	CDIMT H8	CDIMT H9	CDIMT H10	CDIMT H11	CIMT H12

Legend:

The initials in the wells represent the contents of the plate

C - Red Blood Cells

D – Drug that was serially diluted across the row. Row A & B have single drugs, row D to H combined drugs in different ratios

I - Indicator – radio-labeled Hypoxanthine

M – Medium -RPMI 1640 with 10% serum

T - Target the parasite being tested

Plate 1: A template for drug screening assay layout

The seeded plates were exposed to the drugs at 37 ^{0}C in 5 % CO_2 for 24 hours. They were removed from the incubator and pulsed with 25 μl (0.15 μCi) of (^{3}H)-hypoxanthine (specific gravity 1Ci/mMol) isotope solution and incubated again for 18 hours. They were then removed from the incubator and frozen at –20 ^{0}C for at least 48 hours. The plates were then thawed to lyse the red blood cells and then harvested on to fibre filter mats. The plates were harvested using a filter mat harvester (Packard Bioscience instruments). The filter mats were dried at 37 ^{0}C overnight after which scintillation fluid (Microscint^{TM} O) was added and then counted using liquid scintillation counter (Top Count, from Packard Bioscience instruments).

These assays were run in duplicates and repeated twice on different days for each combination against both strains of *P. falciparum* (W2 and D6).

2.3.3 Data Analysis

The scintillation counter (Top Count) gave the data on a spreadsheet as number of radioactive emissions per minute. These raw data were analyzed using Oracle database software (Data Aspects Corporation, California USA), to determine the Inhibitory Concentration at fifty percent (the concentration of drug producing a fifty percent inhibition of uptake of (^{3}H)-hypoxanthine by the intra-erythrocytic parasites). The IC_{50}s were determined for each drug alone and for drugs in fixed concentration and used to calculate the fifty percent Fraction Inhibitory Concentration (IC_{50} of drug A in combination / IC_{50} of drug A alone) of all the drugs and obtained the sums of FIC_{50} of each combination.

Assay exclusion criteria involved were; bacterial contamination, low counts in the control wells, and IC_{50} incorrectly aligned. The software was programmed to analyze the

data (FIC_{50} of drug A and FIC_{50} of drug B) and presented the results as isobolograms, which represented a plane through the center of a three-dimensional dose-response surface (Berenbaum, 1978). Instead of representing individual representative isobolograms (isobolograms per assay), all data points were plotted to represent the complete range of interactions obtained at every fixed ratio of every set of drug combination evaluated.

The data was entered and processed using Microsoft Excel. Sigma plot (version 9.0) and SPSS (version 11.0) were used for statistical calculations and graphics. The means of sums of FIC_{50} of the drugs in combination were calculated and used to determine the level of significance using the unpaired t-distribution test at 5 % level of significance. The means of FIC_{50} of each duplicate assay run in a day were obtained and the data points of all successful sets plotted as a Scatter Plots (Figures 4 to 18). Sigma plot software was used to obtained these parameters and graphics.

The means of FIC_{50} of individual drugs (FIC_{50} of Drug A & FIC_{50} of B), and that of Sum FIC used in the combination assays were obtained using sigma plot software and their association determined by Pearson's correlation coefficient. This association was determined between the isolates, between the AC activators and AC inhibitors by use of SSPS software.

CHAPTER THREE

3. RESULTS

3.1 The IC_{50} for the Anti-malarials and the cAMP modulators

In this study chloroquine resistant strain (W2) and chloroquine sensitive strain (D6) of *Plasmodium falciparum* were exposed to cAMP modulators as single agents. The modulators comprised of two AC inhibitors (9-cyclo and D0689), one AC activator (forskolin) and one PKA activator (A 166). The cAMP modulators used were all membrane permeable.

Table 1 illustrates the means of fifty percent Inhibitory Concentrations (IC_{50}s) for the anti-malarials and the cAMP modulators. The means of IC_{50} of the AC inhibitors on D6 were 17,965.37 ng/ml and 1,866.05 ng/ml while those of W2 were 18,130.32 ng/ml and 2,548.13 ng/ml for 9-cyclo and D0689 respectively. The AC activator had a mean of 24,126.46 ng/ml on D6 and 17,104.51 ng/ml on W2. The PKA activator had a mean of 12,473 ng/ml on D6 and 9883.39 ng/ml on W2.

Unpaired t tests were performed to establish if there were any statistical differences between the anti-malarial activities of the cAMP modulators in the two isolates (D6 and W2). It showed that there was no statistical difference between the anti-malarial activities of the cAMP modulators. The t values of 9-cyclo and D0689 were ($t = 0.078$, $P = 0.9$; $t = 1.25$, $P = 0.2$) and that of forskolin was ($t = 2.06$, $P = 0.07$).

Further t- tests were performed to establish if there were any statistical differences between the anti-malarial activity of the two AC inhibitors and the activators (AC activator and PKA activator). It established that the differences between the anti-malarial

activity of the two AC inhibitors were significant; ($t = 8.34$, $P = 0.000008$) on W2 and ($t = 21.4$, $P = 0.00000002$) on D6. The anti-malarial activity of AC activator and PKA activator had a significant difference only on D6 ($t = 7.68$, $P = 0.00003$).

Table 1: The IC_{50} for anti-malarials and the cAMP modulators

Isolate	Test drug	IC_{50} (ng/ml)	AC modulators	IC_{50} (ng/ml)
D6	CQN N = 6	24.66 ± 5.33	9-cyclo N = 5	17965.37 ± 1489
W2	CQN N = 6	31.64 ± 9.22	9-cyclo N = 6	18,130 ± 4470.56
D6	QN N = 6	57.37 ± 10.34	D0689 N = 5	1866.05 ± 781.24
W2	QN N = 6	93.29 ± 12.68	D0689 N = 6	2548.13 ± 976.08
D6	MQN N = 6	10.70 ± 2.67	Forskolin N = 5	24126.46 ± 965.65
W2	MQN N = 6	16.93 ± 3.49	Forskolin N = 6	17,104.51 ± 7538.6
D6	AQN N = 6	6.43 ± 1.83	A 166 N = 6	12473 ± 3247.5
W2	AQN N = 6	7.42 ± 2.17	A 166 N = 6	9883.39 ± 6324.2
D6	DOXY N = 6	2999.90 ± 1179.89		
W2	DOXY N = 6	5301.99 ± 2074.29		

The values reported are means of IC_{50} and standard deviations for the assays run in duplicates on different days.
Legend: N - No. of assays done , CQN- chloroquine, QN - quinine, MQN - mefloquine
AQN - amodiaquine, Doxy - doxycycline

3.2 The FIC_{50} for the cAMP modulators in combination with the anti-malarials

Table 2 illustrates the means of Sum FIC_{50} on anti-malarials - AC inhibitors combination assays at all the concentration ratios. The combinations were named depending on the drugs used: CQN-9-cyclo for chloroquine and 9-cyclo, CQN-D0689 for

chloroquine and D0689, QN-9-cyclo for quinine and 9-cyclo, QN-D0689 for quinine and D0689, MQN-9-cyclo for mefloquine and 9-cyclo, MQN-D0689 for mefloquine and D0689, AQN-9-cyclo for amodiaquine and 9-cyclo, AQN-D0689 for amodiaquine and D0689, Doxy-9-cyclo for doxycycline and 9-cyclo, Doxy-D0689 for doxycycline and D0689.

The FIC_{50} values varied in the combination assays and within the concentration ratios. The means obtained at dilution concentration ratio of 1:5 for AQN-9-cyclo combination assay showed antagonism with FIC_{50} of 1.58 and 1.22 on D6 and W2. Doxy-D0689 combination was antagonistic with FIC_{50} of 4.08 and 2.03 on D6 and W2 respectively. The rest of the combinations at 1:5 concentration ratios ranged from synergism to additivity.

The means of Sum FIC_{50} of QN-D0689 combination assays were indicative of synergism tending towards additivity at a few concentration ratios on both of the isolates. The AQN-D0689 combinations were indicative of additivity on the D6 isolate across all the concentration ratios (FIC_{50} 1.01 – 0.61). The AQN-9-cyclo combinations indicated antagonism with means of FIC_{50} of 2.12 – 1.22 on both D6 and W2 isolates.

The means of FIC_{50} for Doxy-9-cyclo combination (at the concentration ratio of 3:1) were 1.91 and 2.01, indicating antagonism on both W2 and D6. The means of FIC_{50} for the rest of the concentration ratios were 0.46 – 0.84 on both D6 and W2. This was demonstrated that the AC inhibition complemented doxycycline in these concentration ratios.

Most of the combinations had no statistical differences ($P > 0.05$) on the D6 and W2 isolates. The CQN-D0689 combination, 1:1 ratio ($t = 0.03$, $P = 0.97$), *1:3 ratio ($t =$

3.64, P = 0.0065), 3:1 ratio (t = 0.46, P = 0.65), 1:4 ratio (t = 0.41, P = 0.69), 4:1 ratio (t = 1.26, P = 0.24) and 1:5 ratio (t = 0.44, P = 0.66). The QN- 9-cyclo combination, *1:1 ratio (t = 0.48, P = 0.063), 1:3 ratio (t = 1.45, P = 0.17), 3:1 ratio (t = 1.33, P = 0.20), 1:4 ratio (t = 0.46, P = 0.65), 4:1 ratio (t = 0.98, P = 0.34) and 1:5 ratio (t = 1.33, P = 0.21).

The QN-D0689 combination, *1:1 ratio (t = 2.64, P = 0.02), *1:3 ratio (t = 2.34, P = 0.04), 3:1 ratio (t = 2.1, P = 0.06), *1:4 ratio (t = 2.48, P = 0.03), *4:1 ratio (t = 2.47, P = 0.03) and 1:5 ratio (t = 0.31, P = 0.75). The MQN-9-cyclo combination, 1:1 ratio (t = 0.43, P = 0.67), *1:3 ratio (t = 3.81, P = 0.003), 3:1 ratio (t = 1.43, P = 0.18), *1:4 ratio (t = 4.97, P = 0.0005), 4:1 ratio (t = 1.19, P = 0.25) and *1:5 ratio (t = 3.48, P = 0.006). The MQN-D0689 combination, 1:1 ratio (t = 0.7, P = 0.49) 1:3 ratio (t = 0.02, P = 0.97), 3:1 ratio (t = 0.3, P = 0.76), 1:4 (t = 0.21, P = 0.69) and 1:5 ratio (t = 1.53, P = 0.15).

The AQN-9-cyclo combination, 1:1 ratio (t = 0.87, P = 0.4), 1:3 ratio (t = 0.51, P = 0.61), 3:1 ratio (t = 0.83, P = 0.42), 1:4 (t = 0.03, P = 0.97), 4:1 ratio (t = 0.7, P = 0.49) and 1:5 ratio (t = 1.24, P = 0.23). The AQN-D0689 combination, *1:1 ratio (t = 2.78, P = 0.02), 1:3 ratio (t = 0.16, P = 0.87), *3:1 ratio (t = 5.39, P = 0.0004), 1:4 ratio (t = 1.24, P = 0.24), *4:1 ratio (t = 4.14, P = 0.002) and *1:5 ratio, (t = 4.47, P = 0.001).

The Doxy-9-cyclo combination, 1:1 ratio (t = 0.19, P = 0.85), 1:3 ratio (t = 0.85, P = 0.41), 3:1 ratio (t = 0.2, P = 0.84), 1:4 ratio (t = 0.48, P = 0.63), 4:1 ratio (t = 0.19, P = 0.85) and 1:5 ratio (t = 0.73, P = 0.48). The Doxy-D0689 combination, 1:1 ratio (t = 1.27, P = 0.23), 1:3 ratio (t = 0.72, P = 0.49), 3:1 ratio (t = 1.35, P = 0.21), 1:4 ratio (t = 0.78, P = 0.45), 4:1 ratio (t = 0.16, P = 0.87) and 1:5 ratio, (t = 1.53, P = 0.16).

There were significant differences (P<0.05) in the effect of the AC inhibitors on anti-malarials on D6 and W2 in the following drug combinations: QN–9-cyclo at ratio 1:1, QN – D0689 at ratio 1:1, 1:4, and 4:1; MQN – 9-cyclo at ratio 1:3, 1:4 and 1:5 and AQN – D0689 at ratio 1:1, 3:1, 4:1 and 1:5.

Table 2: AC inhibitors in combination with anti-malarial agents

Isolate	AC inhibitor	$SFIC_{50}$ 1:1	$SFIC_{50}$ 1:3	$SFIC_{50}$ 3:1	$SFIC_{50}$ 1:4	$SFIC_{50}$ 4:1	$SFIC_{50}$ 1:5
D6	CQN-9-cyclo N = 6	1.59± 0.26	1.61± 0.25	1.50± 0.20	1.50± 0.20	1.46± 0.18	0.96± 0.13
W2	N = 6	1.6± 0.15	1.46± 0.16	1.32± 0.43	1.40± 0.18	1.42± 0.13	0.91± 0.13
D6	CQN-D0689 * N = 4	1.47± 0.08	1.48± 0.12	1.73± 0.07	1.03± 0.12	1.18± 0.14	0.75± 0.13
W2	N = 6	1.47± 0.45	1.06± 0.20	1.62± 0.48	1.06± 0.12	1.26± 0.07	0.81± 0.24
D6	QN-9-cyclo N = 6	1.50± 0.14	1.14± 0.59	1.04± 0.55	1.27± 0.36	1.13± 0.21	0.80± 0.28
W2	N = 6	1.44± 0.24	1.55± 0.36	1.37± 0.25	1.35± 0.24	1.27± 0.25	1.03± 0.32
D6	QN-D0689 * N = 6	0.79± 0.26	0.74± 0.32	0.92± 0.24	0.76± 0.35	0.91± 0.23	0.68± 0.16
W2	N = 6	0.51± 0.02	0.42± 0.04	0.7± 0.05	0.39± 0.06	0.66± 0.08	0.71± 0.17
D6	MQN-9 cyclo * N = 6	2.01± 0.86	1.76± 0.28	1.63± 0.75	1.57± 0.23	1.73± 0.34	1.08± 0.27
W2	N = 6	2.18± 0.41	1.23± 0.18	2.10± 0.22	0.97± 0.18	1.92± 0.16	0.66± 0.10
D6	MQN-D0689 N = 6	1.71± 1.21	2.61± 2.87	1.86± 1.68	2.33± 2.37	1.93± 1.75	0.66± 0.16
W2	N = 6	1.32± 0.55	2.65± 2.0	2.12± 1.10	2.6± 1.80	2.27± 1.06	0.83± 0.21
D6	AQN-9-cyclo N = 6	1.52± 0.37	1.60± 0.55	1.70± 0.31	1.49± 0.55	1.63± 0.43	1.58± 0.56
W2	N = 6	1.76± 0.54	1.74± 0.36	2.12± 1.19	1.47± 0.43	1.98± 1.14	1.22± 0.42
D6	AQN-D0689 * N = 5	0.98± 0.08	1 ± 0.14	1.01± 0.07	0.97± 0.03	1.01± 0.04	0.61± 0.10
W2	N = 6	1.16± 0.12	0.98± 0.09	1.28± 0.09	0.91± 0.10	1.33± 0.18	0.86±0.07
D6	Doxy-9-cyclo N = 6	0.84± 0.71	0.81± 0.62	1.91± 0.65	0.81± 0.54	0.69± 0.67	0.56± 0.27
W2	N = 6	0.76± 0.54	0.55± 0.34	2.01± 0.81	0.67± 0.32	0.77± 0.54	0.46± 0.18
D6	Doxy-D0689 N = 6	2.76± 1.12	4.61± 2.68	1.79± 0.54	3.96± 1.95	1.58± 0.60	4.08± 2.57
W2	N = 4	1.99± 0.41	3.61± 0.44	1.39± 0.24	3.17± 0.45	1.64± 0.09	2.03± 0.64

The significant values reported as means of sum FIC_{50} and standard deviations for the assays run in duplicates on different days. N denotes the no. of assays run in each combination. * Denotes the drug combination assays whose effect was statistically different in the 2 isolates tested ($P < 0.05$). Synergy: <1; additivity: 1 and antagonism: >1.

Table 3 illustrates the means of Sum FIC_{50} of the anti-malarials - activators combination assays across the concentration ratios obtained in this study. The combinations were named depending on the drugs used: CQN-FK for chloroquine and forskolin, CQN-A 166 for chloroquine and A 166, QN-FK for quinine and forskolin, QN-A 166 for quinine and A 166, MQN-FK for mefloquine and forskolin, MQN-A 166 for mefloquine and A 166, AQN-FK for amodiaquine and forskolin, AQN-A 166 for amodiaquine and A 166, Doxy-FK for doxycycline and forskolin and Doxy-A 166 for doxycycline and A 166.

The Sum FIC_{50} at concentration ratio of 1:5 showed additivity tending towards antagonism (FIC_{50} 1.18 to 0.80) in most of the combination assays. AQN-A 166 combination indicated synergism (FIC_{50} 0.69) on D6. AQN–A 166 combination assays had means of Sum FIC_{50} that indicated additivity at concentration ratios of 1:3, 3:1, 1:4, 4:1 (FIC_{50} 0.93 to 1.2) and showed antagonism at concentration ratio of 1:1 (FIC_{50} 1.87) on W2. Synergism was observed on this combination at concentration ratio of 1:5 (FIC_{50} 0.69) on W2. Doxy-A 166 combination assays had means of Sum FIC_{50} values ranging from additivity to antagonism across the concentration ratios on W2 (FIC_{50} 0.80 and 1.46). It indicated antagonism in all the concentration ratios on D6 isolate except at 1:5 concentration ratios (FIC_{50} 1.04). The means of Sum FIC_{50} of Doxy-Forskolin combination assays were indicative of additivity at concentration ratio of 3:1 and 4:1 (0.979 and 0.949) on W2. All the other concentration ratios in this combination indicated antagonism.

There were no significant differences between the effects of the drug combinations (anti-malarials – activators) on the two isolates ($P > 0.05$) in most of the

combination assays across the concentration ratios. The CQN-A 166 combination, 1:1 ratio (t = 0.6, P = 0.55), 1:3 ratio (t = 1.65, P = 0.13), 3:1 ratio (t = 1.69, P = 0.12), 1:4 ratio (t = 0.51, P = 0.61) and 4:1 ratio (t = 0.74, P = 0.47). The CQN-FK combination, 1:1 ratio (t = 1.25, P = 0.23), 1:3 ratio (t = 1.81, P = 0.09), 3:1 ratio (t = 1.4, P = 0.18), 1:4 ratio (t = 0.14, P = 0.89), 4:1 ratio (t = 0.15, P = 0.88) and 1:5 ratio (t = 0.009, P = 0.99).

The QN-A 166 combination, 1:4 ratio (t = 1.73, P = 0.11), 4:1 ratio (t = 1.46, P = 0.17) and 1:5 ratio (t = 1.26, P = 0.23). QN-FK combination, 1:1 ratio (t = 0.9, P = 0.38), 1:3 ratio (t = 0.14, P = 0.88), 3:1 ratio (t = 1.37, P = 0.19), 1:4 ratio (t = -0.14, P = 0.88), 4:1 ratio (t = 0.2, P = 0.84) and 1:5 ratio (t = 0.29, P = 0.77).

The MQN-A 166 combination, 1:1 ratio (t = 1.04, P = 0.32), 1:3 ratio (t = 0.08, P = 0.93), 3:1 ratio (t = 1.43, P = 0.18), 1:4 ratio (t = -0.717, P = 0.49), 4:1 ratio (t = 1.67, P = 0.12) and 1:5 ratio (t = 0.08, P = 0.93). MQN-FK combination, 1:1 ratio (t = 0.36, P = 0.72), 1:3 ratio (t = 0.2, P = 0.84), 3:1 ratio (t = 0.29, P = 0.77), 1:4 ratio (t = 0.23, P = 0.81), 4:1 ratio (t = 0.47, P = 0.64) and 1:5 ratio (t = 0.1, P = 0.91).

The AQN-A 166 combination: 1:1 ratio (t = 0.24, P = 0.8) and 4:1 ratio (t = 0.97, P = 0.35). AQN-FK combination, 1:1 ratio (t =1.98, P = 0.08), 1:3 ratio (t = 1.07, P = 0.31), 3:1 ratio (t = 1.79, P = 0.10), 1:4 ratio (t = 0.32, P = 0.75), 4:1 ratio (t = 1.83, P = 0.1) and 1:5 ratio (t = 0.46, P = 0.65). The Doxy-FK combination, 1:3 ratio (t = 1.45, P = 0.17), 3:1 ratio (t = 1.64, P = 0.13), 1:4 ratio (t = 1.87, P = 0.09) and 1:5 ratio (t = 1.56, P = 0.14). The Doxy-A 166 combination, 1:1 ratio (t = 0.59, P = 0.57), 1:3 ratio (t = 1.87, P = 0.11), 3:1 ratio (t = 0.3, P = 0.77), 1:4 ratio (t = 1.95, P = 0.1), 4:1 ratio (t = 0.22, P = 0.83) and 1:5 ratio (t = 1.2, P = 0.28).

There were significant differences between the effect of some of the anti-malarial-activators drug combinations on the isolates at varying concentration ratios ($P < 0.05$). These combinations assays were QN-A 166, ratio 1:1 (t = 3.75, P = 0.003), 1:3 ratio, (t = 2.32, P= 0.04), 3:1 ratio (t = 2.97, P = 0.01); AQN-A 166, 1:3 ratio (t = 4.76, P = 0.0007), 3:1 ratio (t = 2.83, P = 0.01), 1:4 ratio (t = 4.41, P = 0.001), 1:5 ratio (t = 2.244, P = 0.04); Doxy-FK, 1:1 ratio (t = 2.45, P = 0.03) and 4:1 ratio (t = 2.89, P = 0.01).

Table 3: AC and PKA activators in combination with anti-malarials

Isolate	Combination	$SFIC_{50}$ 1:1	$SFIC_{50}$ 1:3	$SFIC_{50}$ 3:1	$SFIC_{50}$ 1:4	$SFIC_{50}$ 4:1	$SFIC_{50}$ 1:5
D6	CQN–FK N = 6	1.31±0.27	1.46±0.35	1.39±0.29	1.31±0.37	1.31±0.28	1.04±0.37
W2	N = 6	1.15±0.15	1.18±0.15	1.21±0.11	1.33±0.21	1.29±0.07	1.03±0.09
D6	CQN-A 166 N = 5	1.67±0.42	1.55±0.26	1.34±0.25	1.45±0.38	1.22±0.22	0.96±0.14
W2	N = 6	1.54±0.20	1.83±0.30	1.56±0.12	1.55±0.17	1.31±0.10	1.15±0.13
D6	QN–FK N = 6	1.43±0.57	1.36±0.37	1.69±0.71	1.33±0.16	1.32±0.52	1.02±0.29
W2	N = 6	1.21±0.13	1.34±0.17	1.28±0.16	1.31± 0.16	1.27±0.16	1.07±0.29
D6	QN-A 166 * N = 6	1.94±0.40	1.62±0.28	1.94±0.53	1.40±0.21	1.55±0.47	0.86±0.18
W2	N = 6	1.31±0.10	1.31±0.16	1.28±0.11	1.22±0.13	1.25±0.14	1.01±0.23
D6	MQN-FK N = 6	2.37±0.66	2.0±0.56	2.07±0.35	1.69±0.34	1.93±0.38	1.16±0.36
W2	N = 5	2.24±0.44	1.95±0.44	2.14±0.39	1.65±0.24	1.83±0.24	1.18±0.27
D6	MQN-A 166 N = 6	2.26±0.68	1.84±0.36	2.33±1.06	1.72±0.33	2.21±1.09	1.13±0.27
W2	N = 5	2.65±0.50	1.86±0.26	3.19±0.90	1.58±0,29	3.57±1.59	1.11±0.22
D6	AQN–FK N = 4	0.94±0.12	0.92±0.11	0.84±0.12	0.92±0.35	0.86±0.28	0.86±0.24
W2	N = 6	1.13±0.15	1.20±0.30	1.14±0.30	0.99±0.33	1.2±0.28	0.80±0.11
D6	AQN-A 166 * N = 6	1.8±0.46	1.8±0.2	1.60±0.25	1.78±0.27	1.35±0.23	1.01±0.19
W2	N = 4	1.87±0.28	1.20±0.30	1.14±0.30	0.93±0.53	1.2±0.28	0.69±0.39
D6	Doxy-FK * N = 6	1.66±0.25	1.69±0.33	1.55±0.83	1.60±0.33	1.22±0.15	1.34±0.42
W2	N = 6	1.38±0.12	1.47±0.14	0.98±0.16	1.32±0.15	0.95±0.16	0.97±0.37
D6	Doxy-A 166 N = 4	1.63±0.27	1.70±0.33	1.48±0.26	1.46±0.14	1.39±0.26	1.04±0.37
W2	N = 3	1.80±0.47	1.22±0.31	1.57±0.55	0.99±0.33	1.46±0.53	0.80±0.11

The significant values reported as means of sum FIC_{50} and standard deviations for the assays run in duplicates on different days. N denotes the no. of assays run in each combination. *denotes the drug combination assays whose effect was statistically different on the two isolates tested ($P < 0.05$). Synergy: <1; additivity: =1 and antagonism: >1.

Table 4 represents t tests results performed to establish if there were any statistical differences between the effect of anti-malarial activity of anti-malarial drugs in combination with AC inhibitors between the two AC inhibitors used (9-cyclo and D0689). They revealed that there were statistical differences in the anti-malarial activity at different concentration ratios on the D6 and W2 isolates.

The differences between the effect of AC inhibitors tested on W2 were established on: CQN at ratio 1:3 ($t = 3.66$, $P = 0.004$), 1:4 ratio ($t = 3.59$, $P = 0.004$) and ratio 4:1 ($t = 2.55$, $P = 0.02$); QN ratio 1:1 ($t = 9.21$, $P = 0.0000003$), 1:3 ratio ($t = 7.49$, $P = 0.00002$), 3:1 ratio ($t = 6.37$, $P = 0.00008$); 1:4 ratio ($t = 9.35$, $P = 0.000002$); 4:1 ratio ($t = 5.5$, $P = 0.0002$); MQN ratio 1:1 ($t = 3.02$, $P = 0.01$), ratio 1:4 ($t = 2.2$, $P = 0.05$); AQN 1:1 ratio ($t = 2.35$, $P = 0.04$), 1:3 ratio ($t = 4.37$, $P = 0.001$), ratio 1:4 ($t = 2.83$, $P = 0.02$) and Doxy ratio 1:1 ($t = 3.79$, $P = 0.005$) ratio 1:3 ($t = 12.29$, $P = 0.000001$), ratio 1:4 ($t = 10.27$, $P = 0.000006$), ratio 4:1 ($t =3.11$, $P = 0.01$) and ratio 1:5 ($t = 5.8$, $P = 0.0004$), (table 4).

The differences between the effect of AC inhibitors tested on D6 was established on: CQN at ratio 1:4 ($t = 4.09$, $P = 0.003$), 4:1 ratio ($t = 2.56$, $P = 0.03$), ratio 1:5 ($t = 2.32$, $P = 0.04$); QN ratio 1:1 ($t = 5.73$, $P = 0.0001$), ratio 1:4 ($t = 2.45$, $P =0.03$); MQN ratio 1:5 ($t = 3.14$, $P = 0.01$) ; AQN ratio 1:1 ($t = 3.39$, $P = 0.006$), ratio 1:3 ($t =2.54$, $P = 0.02$), ratio 3:1 ($t = 5.24$, $P = 0.0003$), ratio 1:4 ($t = 2.27$, $P = 0.04$), ratio 4:1 ($t = 3.46$, $P = 0.006$), ratio 1:5 ($t = 4.15$, $P = 0.001$) and Doxy ratio 1:1 ($t = 3.01$, $P = 0.01$);1:3 ratio ($t = 2.72$, $P = 0.01$) and ratio 1:5 ($t = 2.66$, $P = 0.02$), (table 4).

Table 4: T-values obtained by AC inhibitors in combination with various anti-malarials

Ratio conc.	T - test	CQN on W2	CQN on D6	QN on W2	QN On D6	MQN on W2	MQN on D6	AQN on W2	AQN on D6	DOXY on W2	DOXY on D6
1:1	T value	0.69	0.81	9.21	5.73	3.02	0.49	2.35	3.39	3.79	3.01
	P value	*0.5	0.43	*3 x 10^{-7}	*1 x 10^{-4}	*0.01	0.63	*0.04	*0.006	*0.005	*0.01
1:3	T value	3.66	0.93	7.49	1.46	1.66	0.71	4.37	2.54	12.29	2.72
	P value	*0.004	0.37	*2 x 10^{-5}	0.17	0.12	0.48	*0.001	*0.02	*1x10^{-6}	*0.02
3:1	T value	1.12	2.17	6.37	0.49	0.04	0.3	1.54	5.24	1.44	0.3
	P value	0.28	0.061	*8 x 10^{-5}	0.62	0.96	0.76	0.15	*3x10^{4}	0.18	0.76
1:4	T value	3.59	4.09	9.35	2.45	2.2	0.78	2.83	2.27	10.27	3.09
	P value	*0.004	*0.003	*2 x 10^{-4}	*0.03	*0.05	0.45	*0.02	*0.04	*6x10^{-6}	*0.01
4:1	T value	2.55	2.56	5.5	1.72	0.8	0.26	1.22	3.46	3.11	2.17
	P value	*0.02	*0.03	*2 x 10^{-4}	0.11	0.44	0.79	0.24	*0.006	*0.01	0.06
1:5	T value	0.83	2.32	2.1	0.85	1.71	3.14	1.9	4.15	5.8	2.66
	P value	0.42	*0.04	0.06	0.41	0.11	*0.01	0.08	*0.001	*4x10^{-4}	*0.02

Legend: * represents the significant P values when $P < 0.05$.

Table 5 represents the t test results performed to establish if there were any statistical differences between the effect of anti-malarial activity of anti-malarials in combination with AC activator (forskolin) and PKA activator (A166). There were statistical differences in the effect of anti-malarial activity in most of the anti-malarials when in combination with Forskolin and anti-malarials when in combination with A166. The anti-malarials at different concentration ratios that showed statistical differences on W2 are: CQN, ratio 1:1 (t = 3.56, P = 0.006), ratio 1:3 (t = 4.62, P = 0.001), ratio 3:1 (t = 4.86, P =0.0008); MQN, ratio 3:1 (t = 2.38, P = 0.04), ratio 4:1 (t = 2.4, P = 0.04); AQN, ratio 1:3 (t =3.26, P = 0.01), ratio 1:4 (t =2.38, P = 0.04), ratio 1:5 (t = 2.86, P = 0.02) and Doxy at ratios, 3:1 (t = 2.54, P = 0.03) and 4:1 (t = 2.27, P = 0.05), (table 5).

There were statistical differences (P<0.05) between the effect of AC activation and PKA activation on anti-malarials tested on D6 only on AQN at the following concentration ratios; 1:1(t = 3.49, P = 0.008), 1:3 (t = 7.93, P = 0.00004), 3:1 (t = 5.39, P = 0.0006), 1:4 (t = 2.78, P = 0.01) and 4:1 (t = 2.93, P = 0.01), (table 5).

Table 5: T-values obtained by various AC activator and PKA activator in combination with various anti-malarials

Ratio conc.		CQN on W2	CQN on D6	QN on W2	QN on D6	MQN on W2	MQN on D6	AQN on W2	AQN on D6	DOXY on W2	DOXY on D6
1:1	T value	3.56	1.25	1.46	1.79	1.36	-0.26	0.96	3.49	2.18	0.19
	P value	*0.006	0.23	0.17	0.1	0.2	0.79	0.36	*0.008	0.06	0.85
1:3	T value	4.62	0.45	0.29	1.36	0.39	-0.61	3.26	7.93	1.68	0.02
	P value	*0.001	0.65	0.77	0.2	0.7	0.55	*0.01	*4x10^{-5}	0.13	0.98
3:1	T value	4.86	0.29	0.06	0.68	2.38	0.56	2.13	5.39	2.54	0.17
	P value	*0.0008	0.77	0.95	0.5	*0.04	0.58	0.07	*6x10^{-4}	*0.03	0.86
1:4	T value	1.77	0.65	1.01	0.68	0.39	0.14	2.38	2.78	1.76	0.77
	P value	0.1	0.52	0.33	0.5	0.7	0.89	*0.04	*0.01	0.12	0.45
4:1	T value	0.29	0.57	0.23	0.8	2.4	0.58	1.81	2.93	2.27	1.32
	P value	0.77	0.57	0.81	0.44	*0.04	0.56	0.11	*0.01	*0.05	0.22
1:5	T value	1.62	0.47	0.36	1.12	0.43	0.18	2.86	1.1	1.04	1.1
	P value	0.138	0.64	0.72	0.28	0.67	0.85	*0.02	0.3	0.33	0.3

Legend: * represents the significant P values when $P < 0.05$.

3.3 Scatter plots representing a range of activity exhibited by the anti-malarial - cAMP modulators combinations

Figures 4 to 18 illustrate the scatter plots whose variables were obtained from the FIC_{50} of anti-malarial and cAMP modulators at different concentration ratios on both D6 and W2 isolates. The means of each set of each successful duplicate assay is presented. Isobolograms can be visually fit through the data points. A concave

isobologram is consistent with synergy, a convex one is consistent with antagonism and a straight line is consistent with additivity. Axes are FIC_{50}s normalized to 1.

The FIC_{50} of chloroquine and AC activator were used to plot the scatter plots in figure 4 obtained by chloroquine – AC activator (forskolin) combination assays on W2 and D6. It shows a convex picture illustrating antagonism with exception of two points on the additivity line for W2 and two other points for D6 assays below the line illustrating synergy. The general picture can be described as antagonistic. Most of the points are seen to hang on the upper Y-axis, illustrating a high negative relationship between the FIC_{50} for chloroquine and FIC_{50} for forskolin.

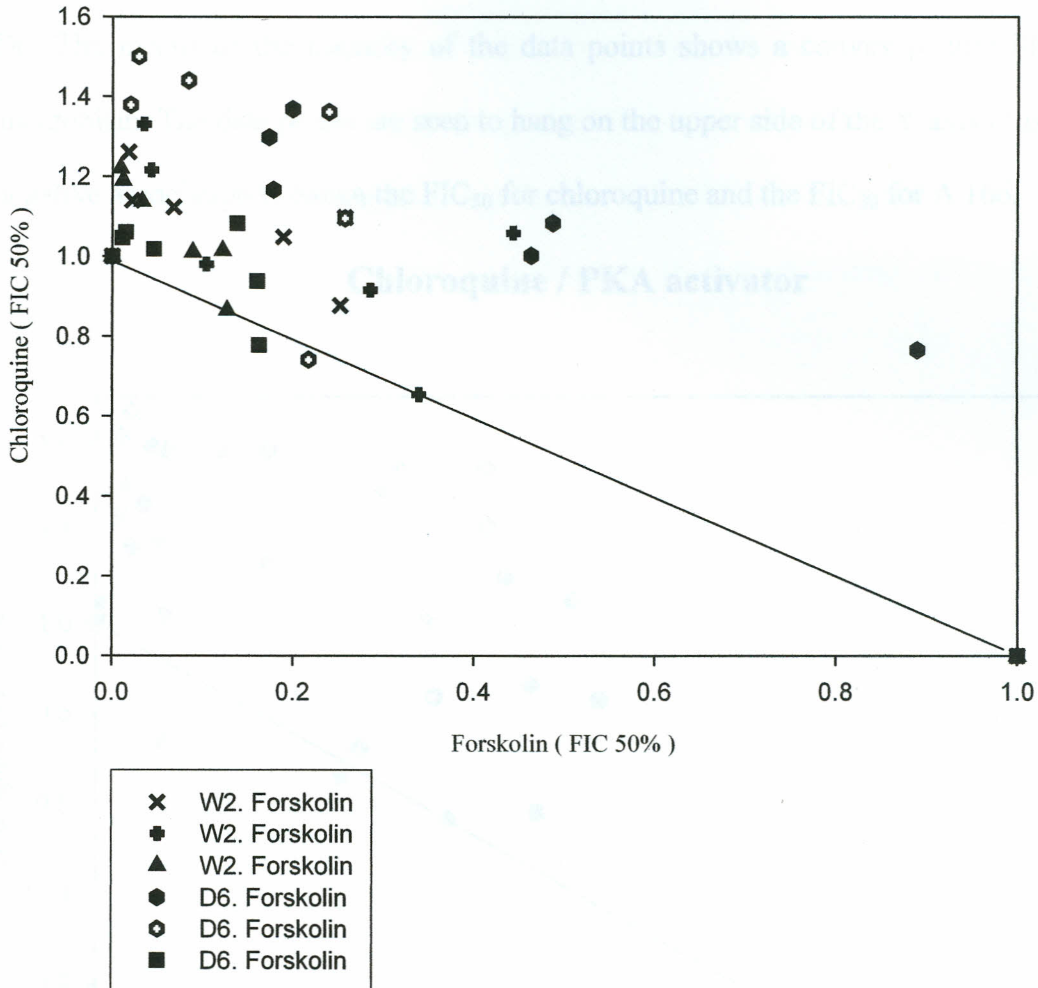


Figure 4: Forskolin and chloroquine FIC_{50} at various concentration ratios

The FIC_{50} of chloroquine and PKA activator were used to plot the scatter plots in Figure 5 obtained by chloroquine – PKA activator (A 166) combinations on both W2 and D6. The means of the majority of the data points shows a convex picture illustrating antagonism. The data points are seen to hang on the upper side of the Y-axis illustrating a negative association between the FIC_{50} for chloroquine and the FIC_{50} for A 166.

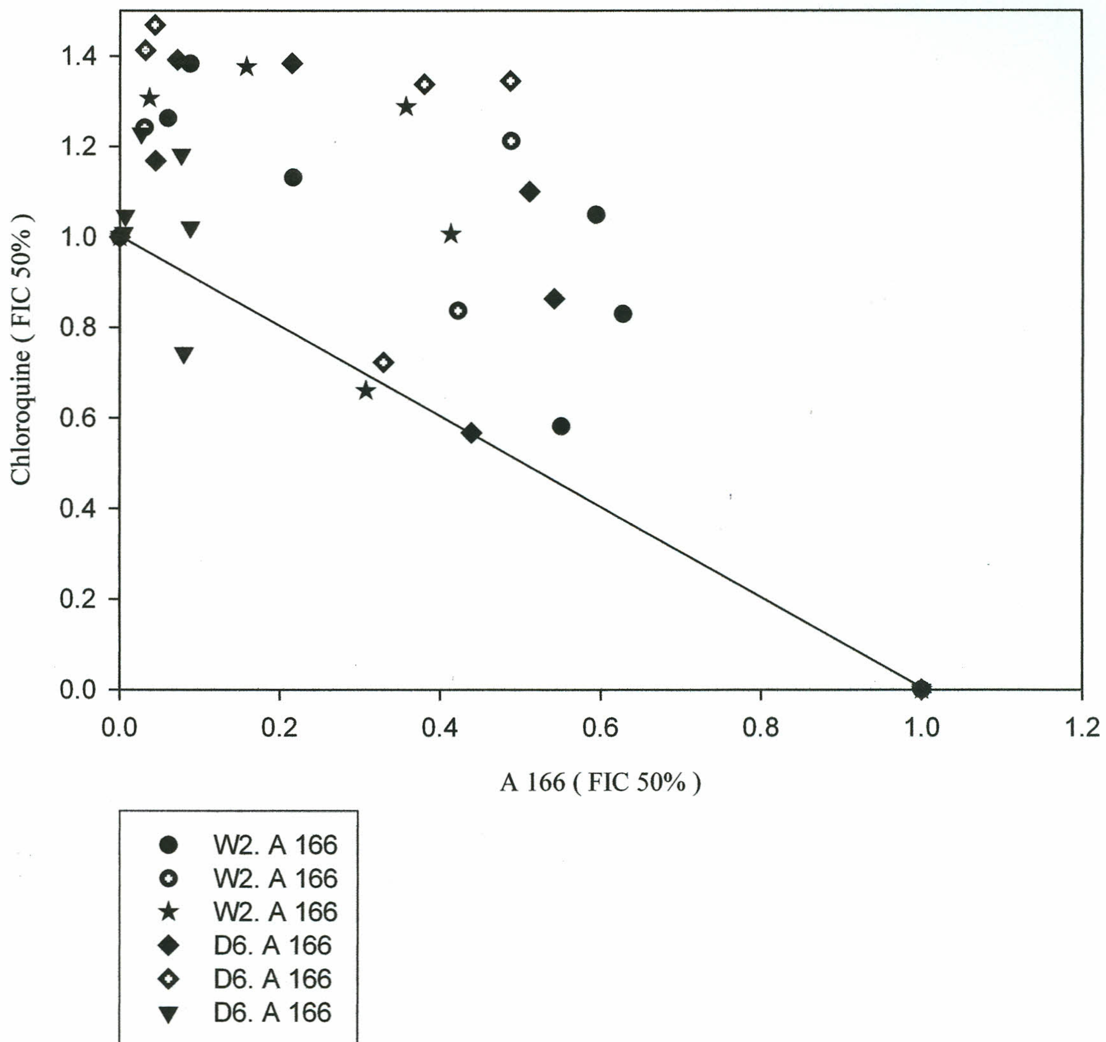


Figure 5: A 166 and chloroquine FIC_{50} at various concentration ratios

The FIC_{50} of chloroquine and AC inhibitors (9-cyclo and D0689) were used to plot the scatter plots in Figure 6 obtained by chloroquine – AC inhibitors combination assays on both D6 and W2 isolates. The majority of the data points have a shift to the left of the line of additivity illustrating antagonism. There is a slight shift to the left indicating synergism and a few data points along the line of additivity. The different concentration ratios have exhibited differences, an indication of dose dependent effect of the AC inhibitors on chloroquine.

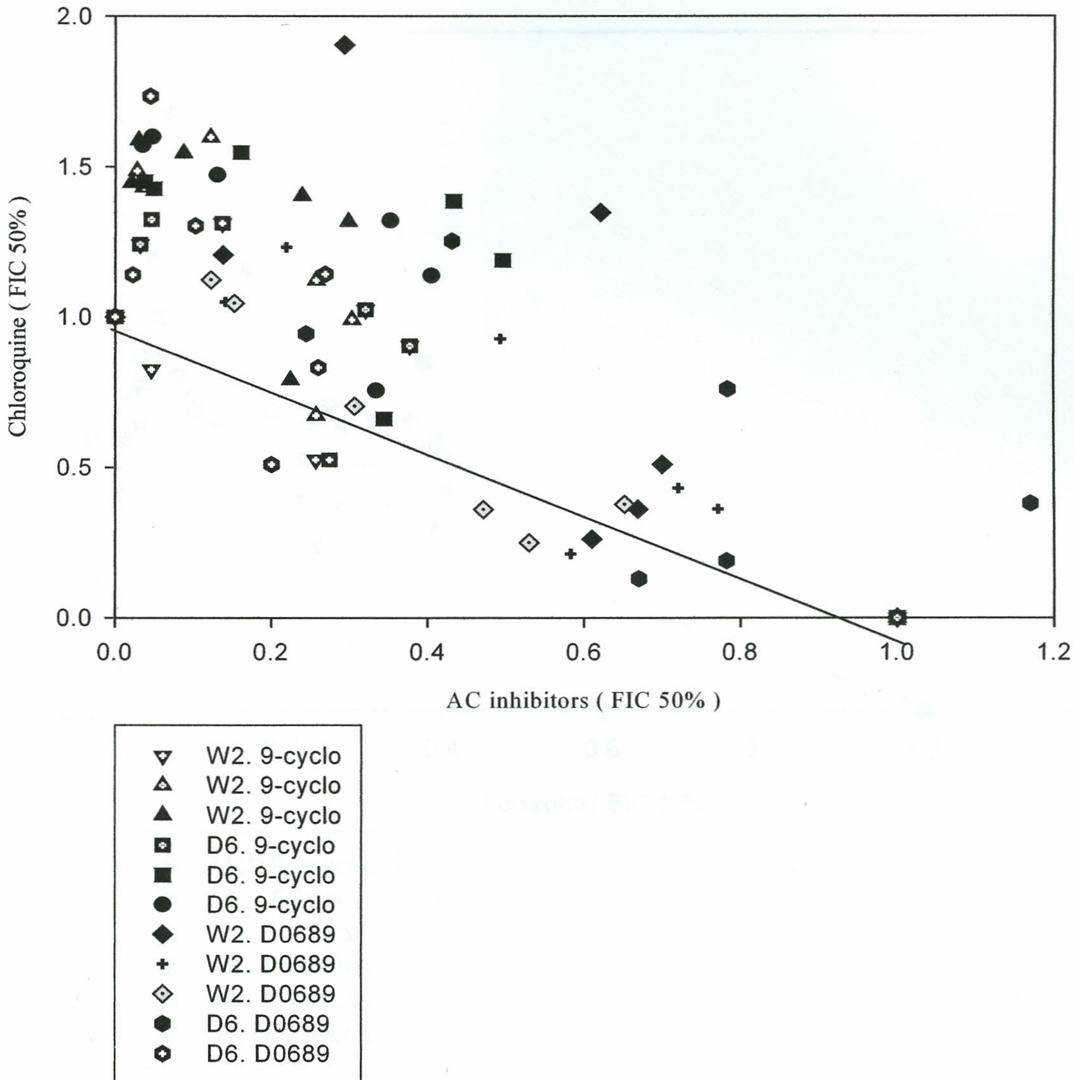


Figure 6: Adenylyl inhibitors and chloroquine FIC_{50} at various concentration ratios

The FIC_{50} of quinine and AC activator were used to plot the scatter plots in the Figure 7 obtained by quinine – AC activator (Forskolin) combination assays on both D6 and W2 isolates. It is a convex picture illustrating antagonism with all the point hanging on the upper side of Y-axis along the line of additivity. This indicates a negative association between quinine and AC activator.

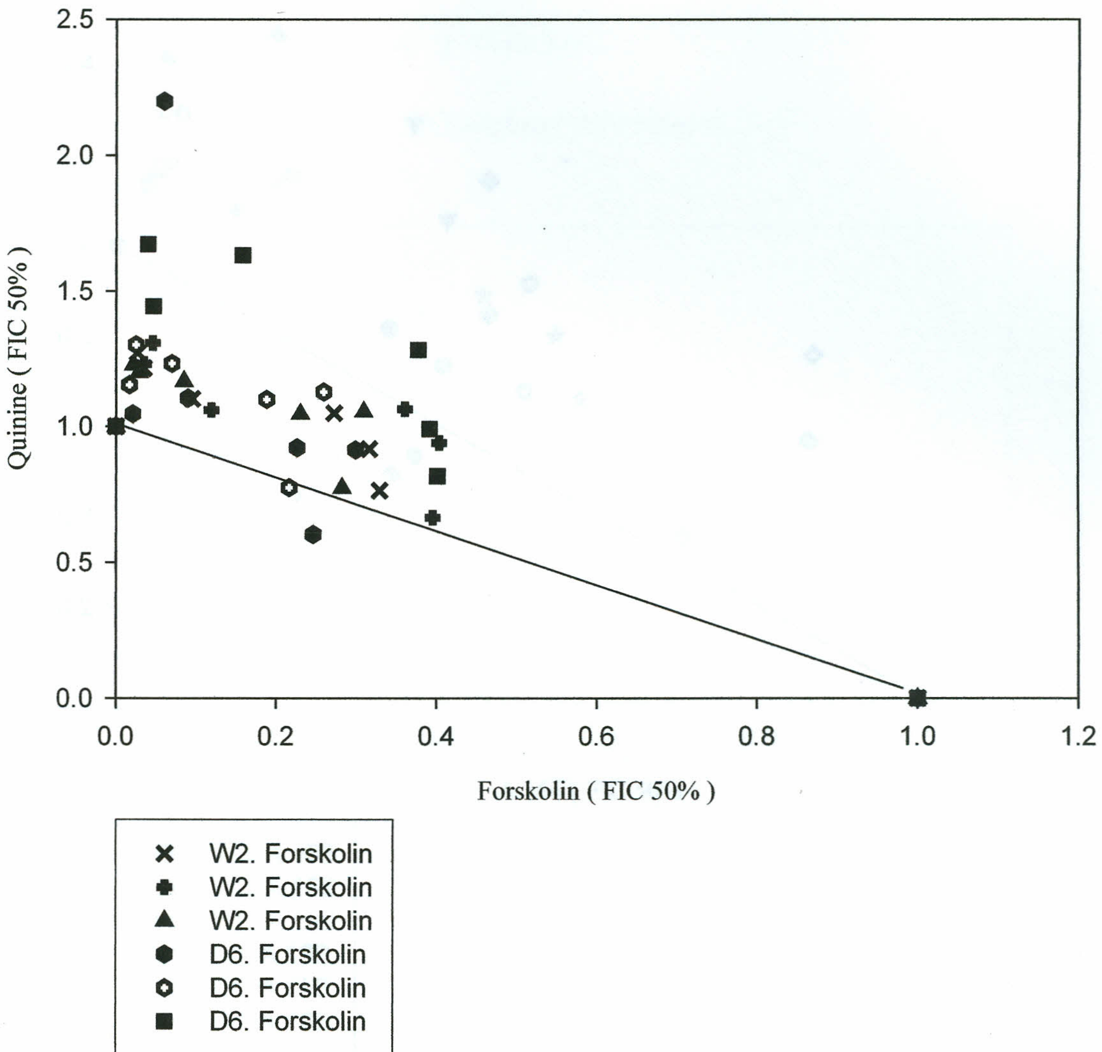


Figure 7: Forskolin and quinine FIC_{50} at various concentration ratios

The FIC_{50} of quinine and PKA activator were used to plot the scatter plots in Figure 8 obtained by quinine – PKA activator (A 166) combination assays on both D6 and W2 isolates. The general picture is a convex illustrating antagonism in this combination at all the concentration ratios.

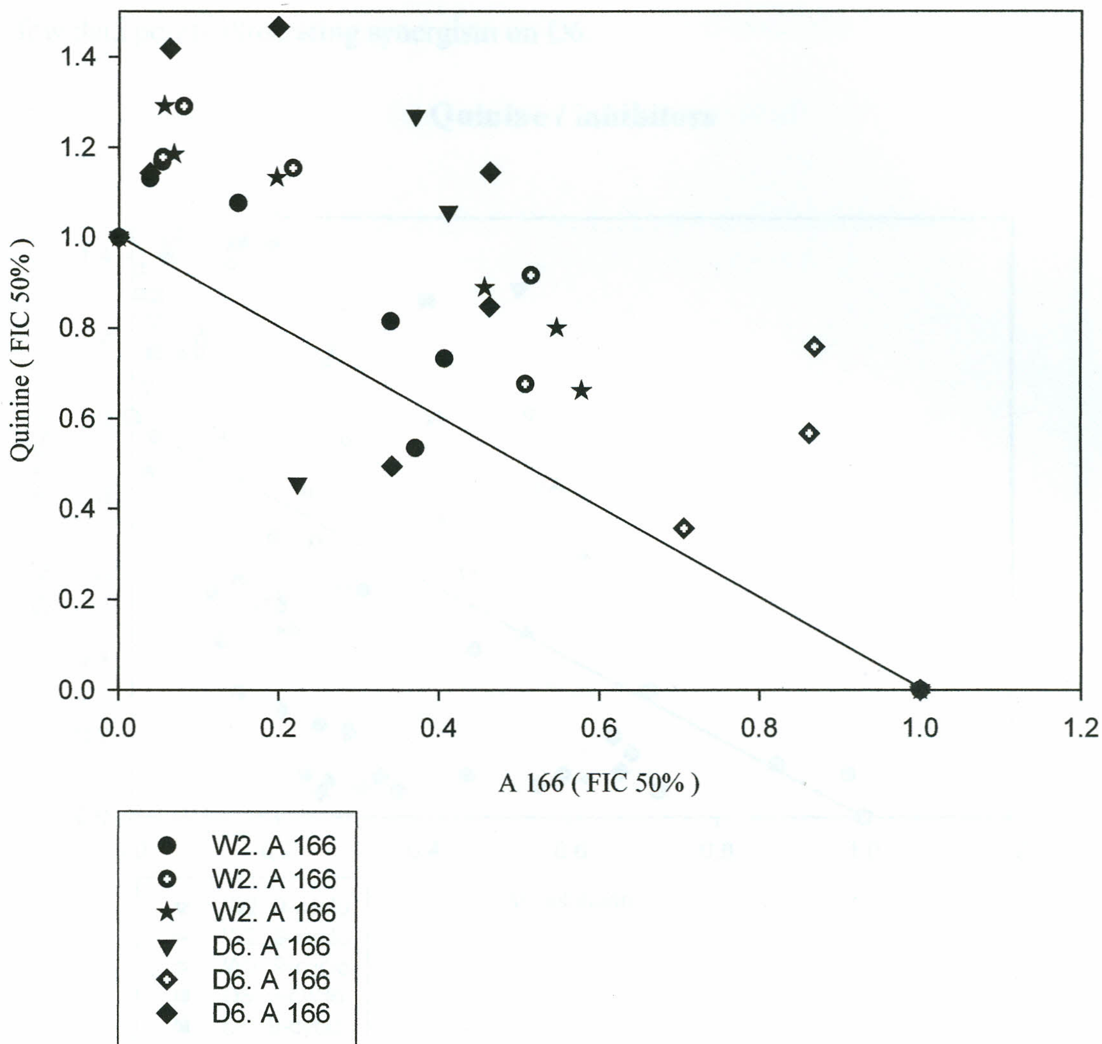


Figure 8: A 166 and quinine FIC_{50} at various concentration ratios

The FIC_{50} of quinine and AC activator were used to plot the scatter plots in Figure 9 obtained by quinine – AC inhibitors (9-cyclo and D0689) combination assays on both D6 and W2 isolates. The general picture shows an even distribution of the data points across the line of additivity. The inhibitors in this assay portray differences in the effect of quinine on the isolates. Most of the points of D0689 lie on the left of the additivity

line. This illustrates synergism with a few points showing additivity on both D6 and W2. Notably the majority of the points of 9-cyclo mainly illustrate antagonism except for a few data points illustrating synergism on D6.

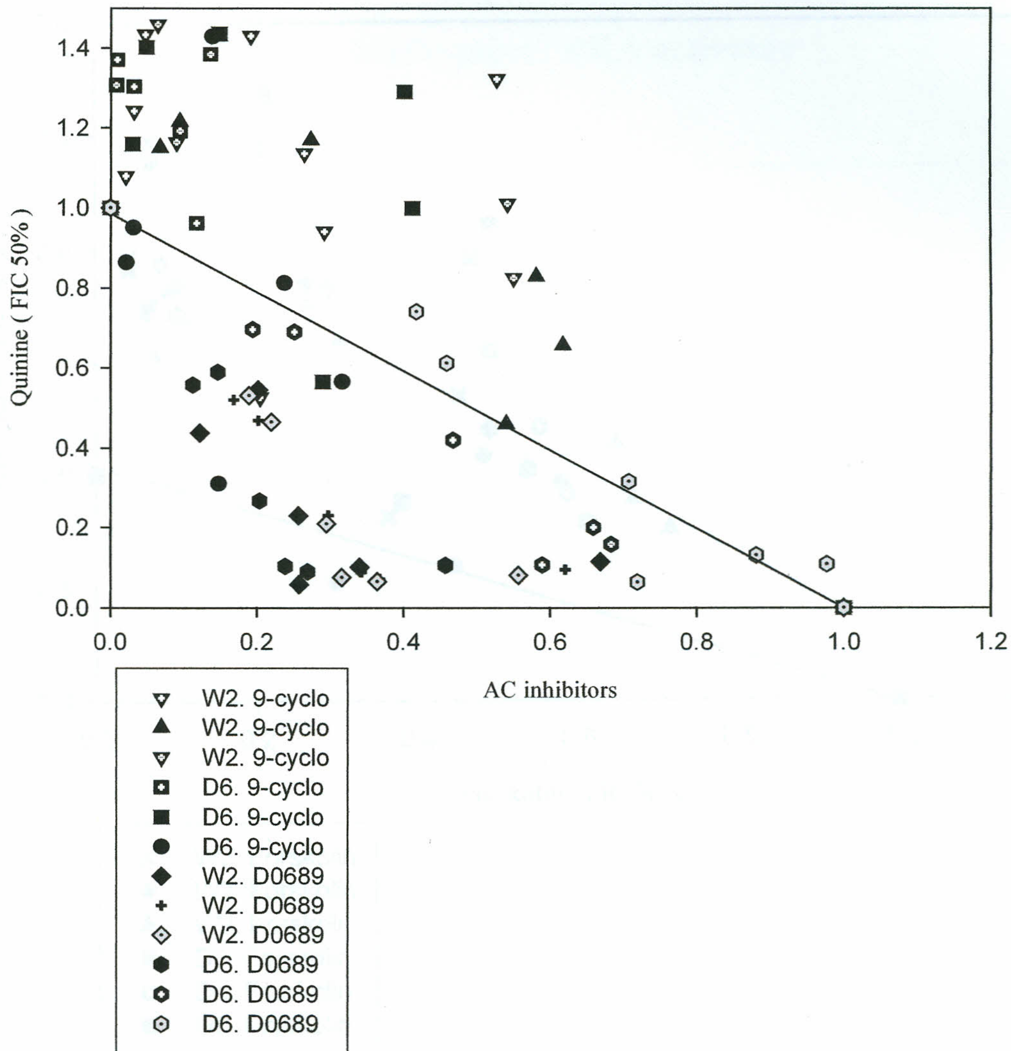


Figure 9: AC inhibitors and quinine FIC_{50} at various concentration ratios

The FIC_{50} of mefloquine and AC activator were used to plot the scatter plots in Figure 10 obtained by mefloquine – AC activator (forskolin) combination assays on both D6 and W2 isolates. The general picture is indicative of antagonism.

Figure 10: Forskolin and mefloquine FIC_{50} at various concentration ratios

The FIC_{50} of mefloquine and PKA activator were used to plot the scatter plots in Figure 11 obtained by mefloquine – PKA activator (A 166) combination assays on both D6 and W2 isolates. The general picture is a convex illustrating antagonism for most of the concentration ratios with exception a few data points that have a shift to the left, tending towards synergism.

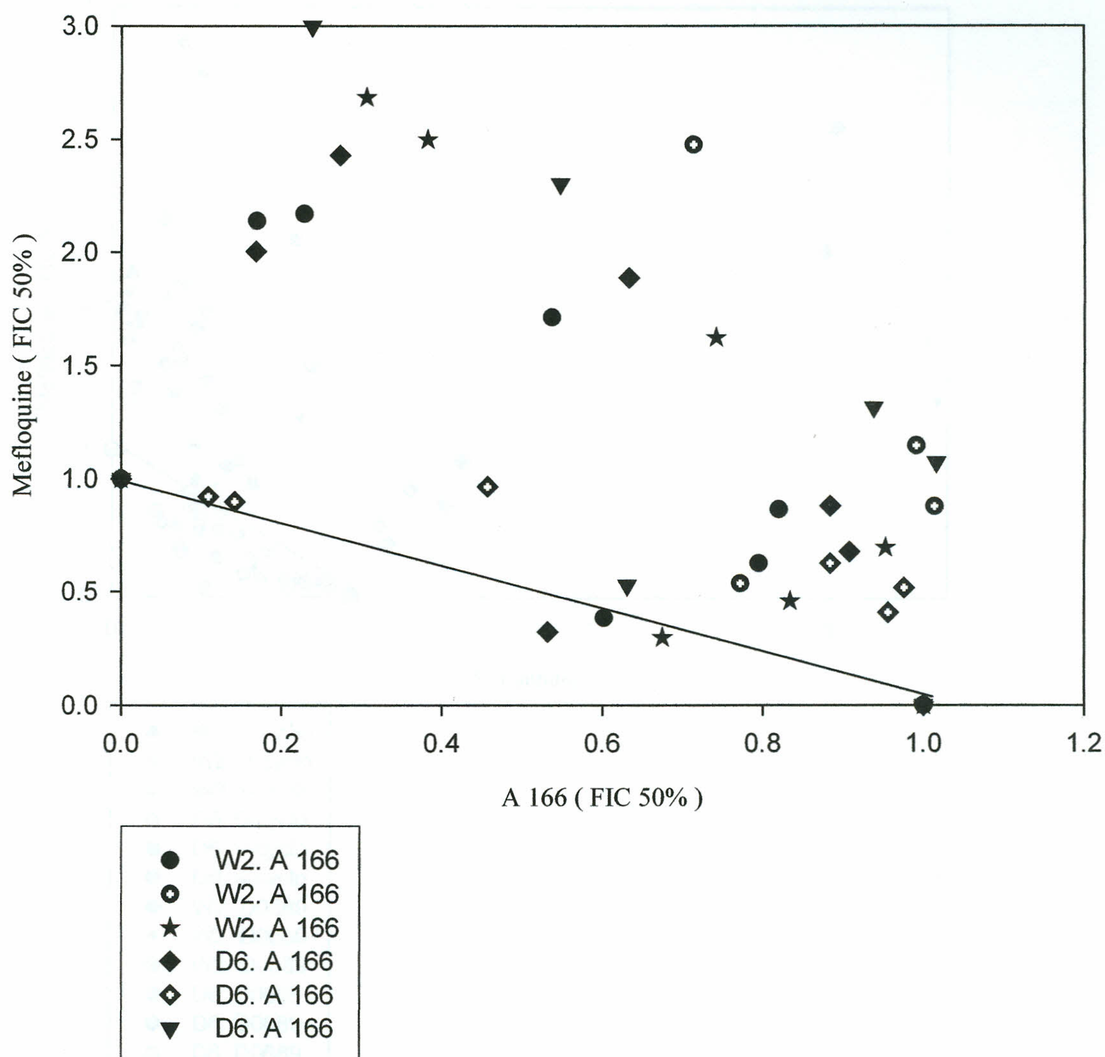
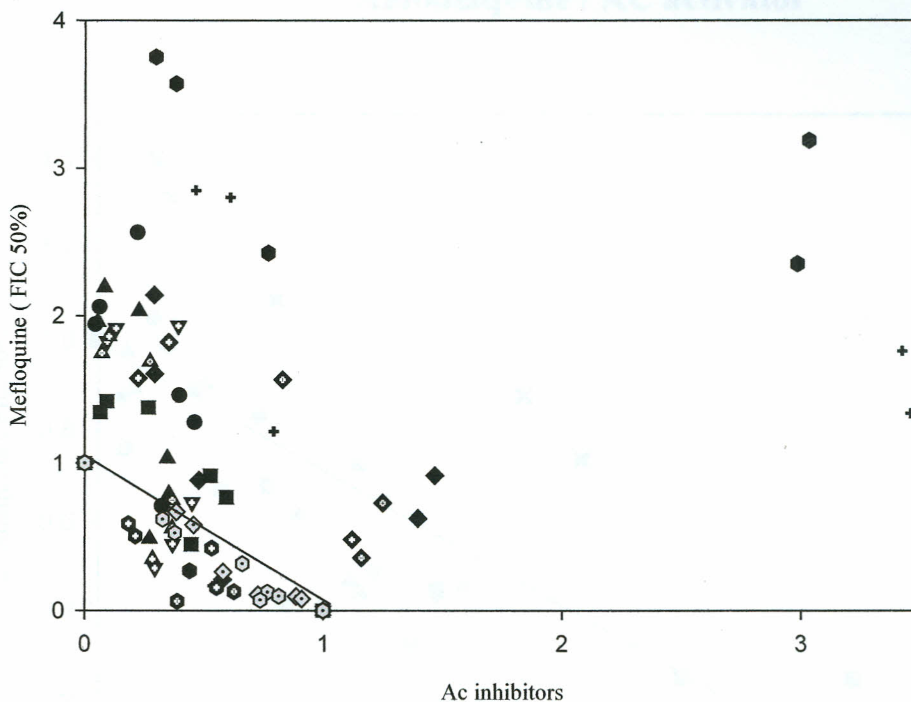


Figure 11: A 166 and mefloquine FIC_{50} at various concentration ratios

The FIC_{50} of mefloquine and AC inhibitors were used to plot the scatter plots in Figure 12 obtained by mefloquine – AC inhibitors combination assays on both D6 and W2 isolates. The data points are widely distributed indicating antagonism tending towards additivity then a shift to the left indicating synergism. The two inhibitors used are shown to have different effects on mefloquine when tested against D6 and W2.

Mefloquine / AC inhibitors



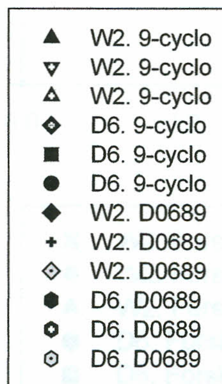


Figure 12: Adenylyl inhibitors and mefloquine FIC_{50} at various concentration ratios

The FIC_{50} of amodiaquine and AC activator were used to plot the scatter plots in Figure 13 obtained by amodiaquine – AC activator (forskolin) combination assays on both D6 and W2 isolates. These isolates have been affected differently upon exposure to the drugs. Most of the D6 data points are indicative of synergism tending towards additivity whereas most of the W2 data points are indicative of antagonism tending towards synergism.

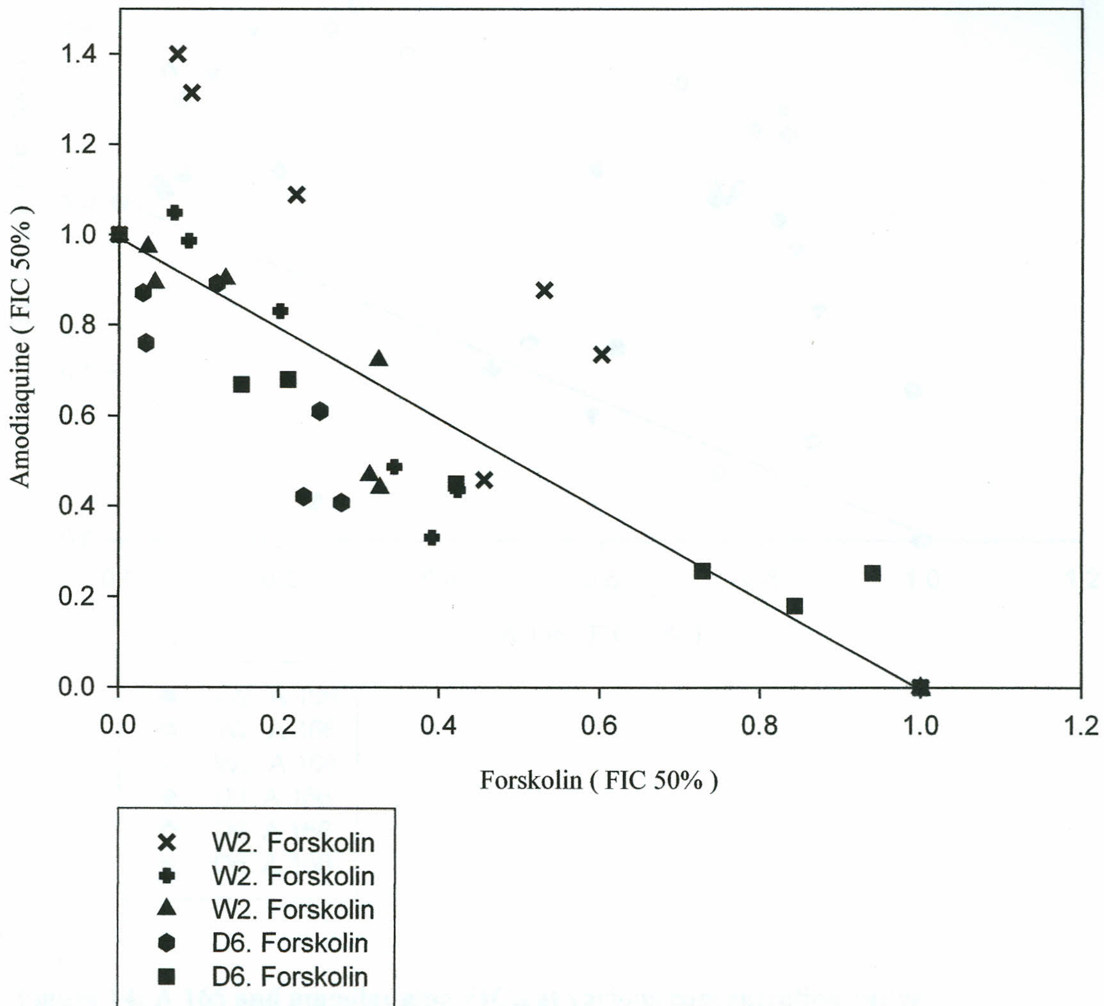


Figure 13: Forskolin and amodiaquine FIC_{50} at various concentration ratios

The FIC_{50} of amodiaquine and PKA activator were used to plot the scatter plots in Figure 14 obtained by amodiaquine – PKA activator (A 166) combination assays on both D6 and W2 isolates. The general picture is a convex illustrating antagonism with a few data points tending towards additivity.

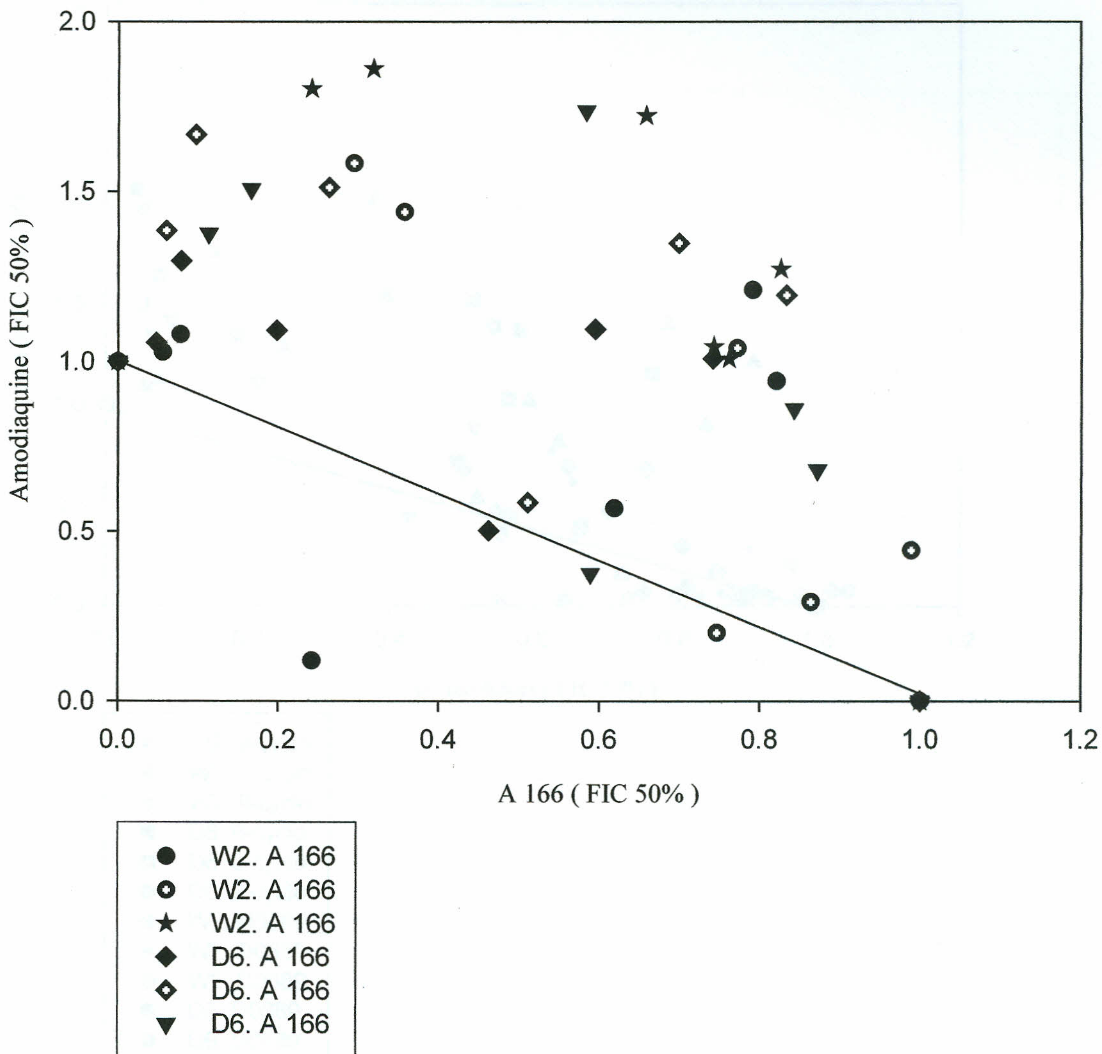


Figure 14: A 166 and amodiaquine FIC_{50} at various concentration ratios

The FIC_{50} of amodiaquine and AC inhibitors were used to plot the scatter plots in Figure 15 obtained by amodiaquine – AC inhibitors (9-cyclo and D0689) combination assays on both D6 and W2 isolates. The majority of the data points illustrate antagonism tending towards additivity and synergism.

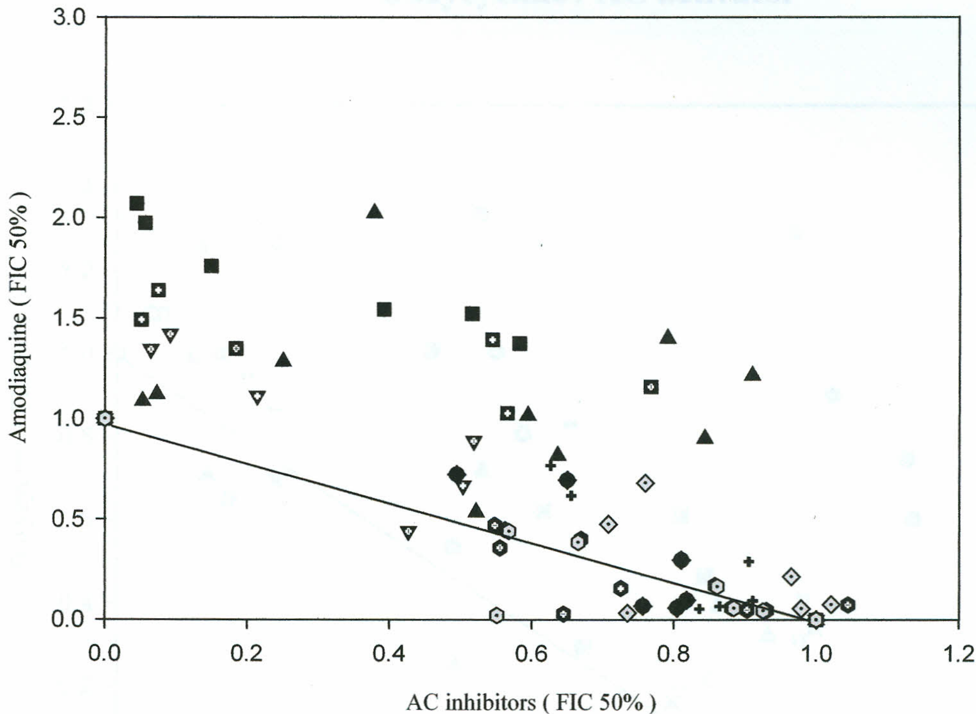


Figure 15: Adenylyl cyclase inhibitors and amodiaquine FIC_{50} at various concentration ratios

The FIC_{50} of doxycycline and AC activator were used to plot the scatter plots in Figure 16 obtained by doxycycline – AC activator (forskolin) combination assays on both D6 and W2 isolates. The general picture is a convex indicative of an antagonistic effect of AC activator on anti-malarial on both isolates used with exception of a few data points on D6 which are indicative of synergism.

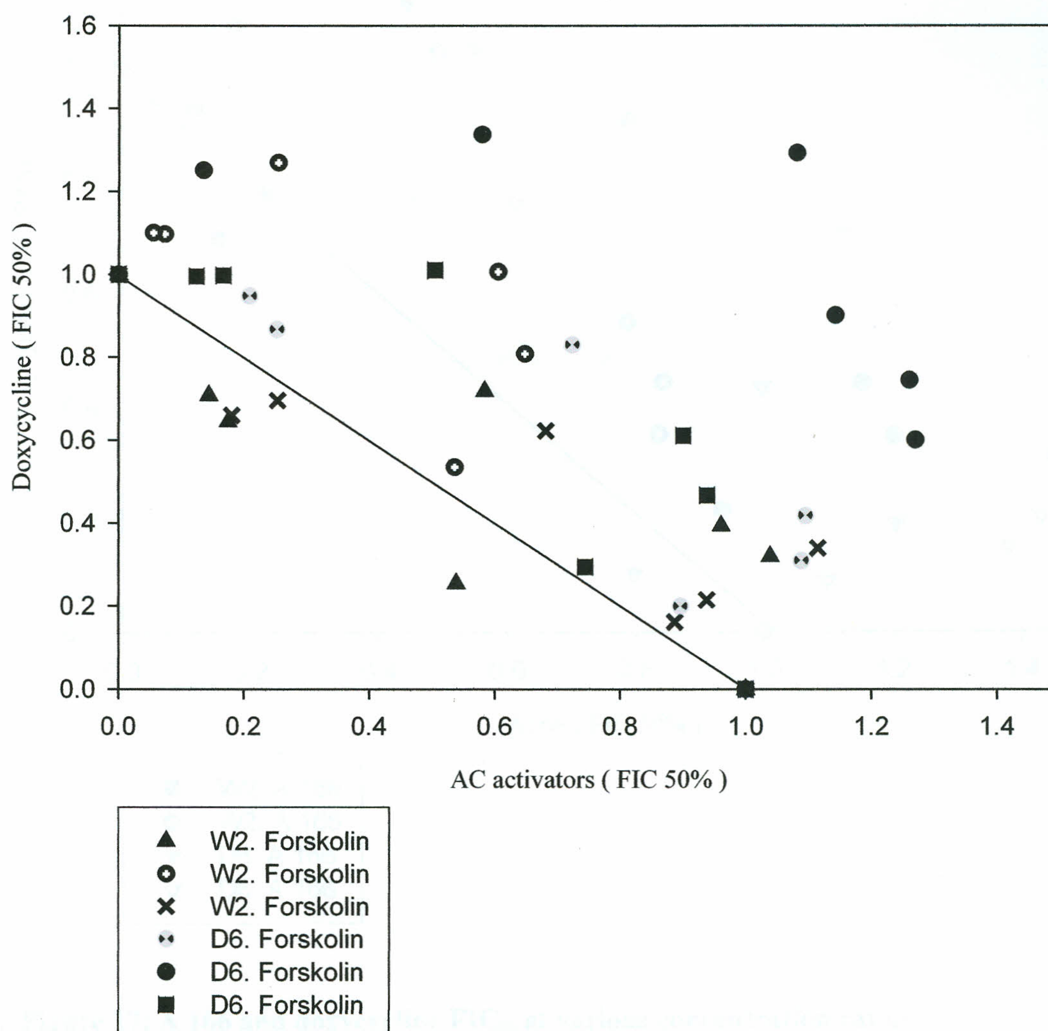


Figure 16: Forskolin and doxycycline FIC_{50} at various concentration ratios

The FIC_{50} of doxycycline and PKA activator were used to plot the scatter plots in Figure 17 obtained by doxycycline – PKA activator (A 166) combination assays on both D6 and W2 isolates. The general picture is indicative of antagonism.

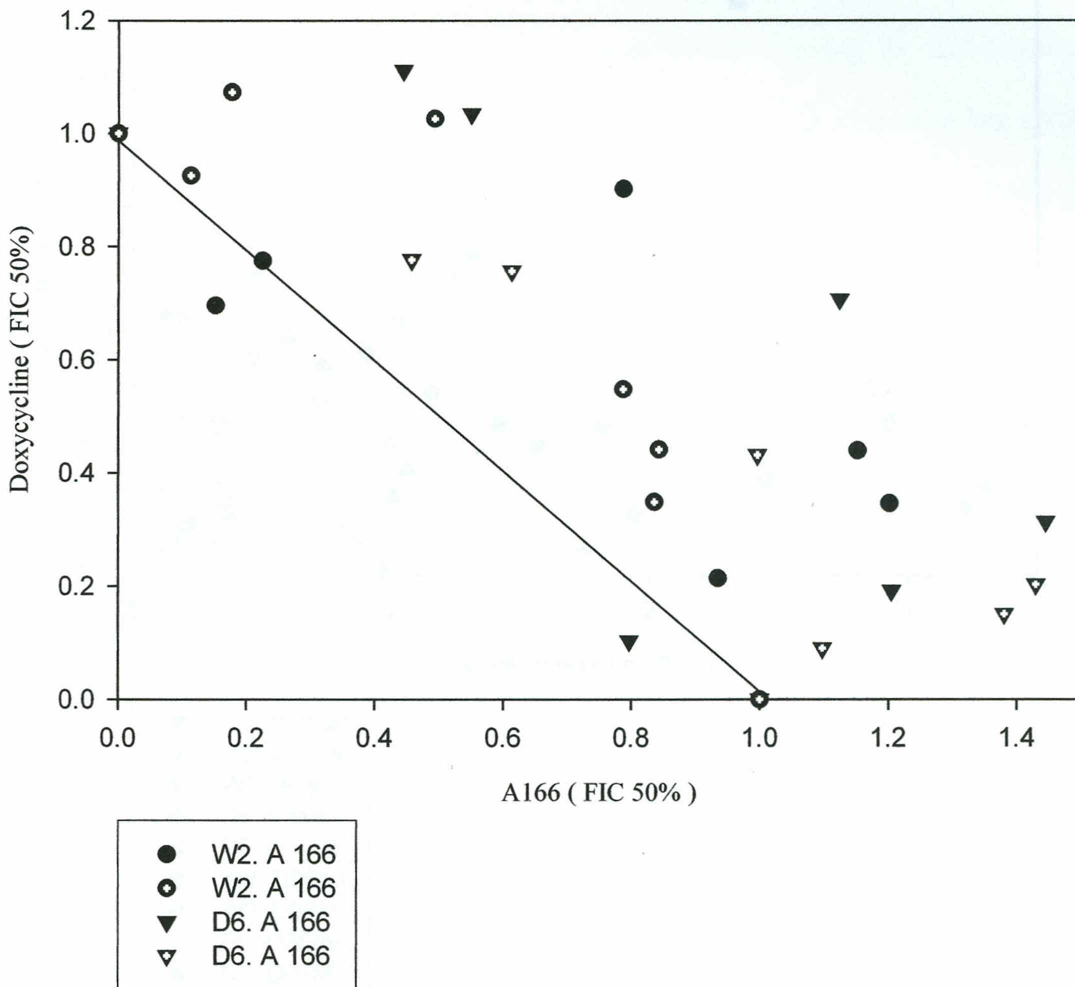


Figure 17: A 166 and doxycycline FIC_{50} at various concentration ratios

The FIC_{50} of doxycycline and AC inhibitors were used to plot the scatter plots in Figure 18 obtained by doxycycline – AC inhibitors (9-cyclo and D0689) combination

assays on both D6 and W2 isolates. The effect of 9-cyclo on doxycycline to W2 is seen to be synergistic with most of the data points below the additive line. The rest of the data points are indicative of antagonism.

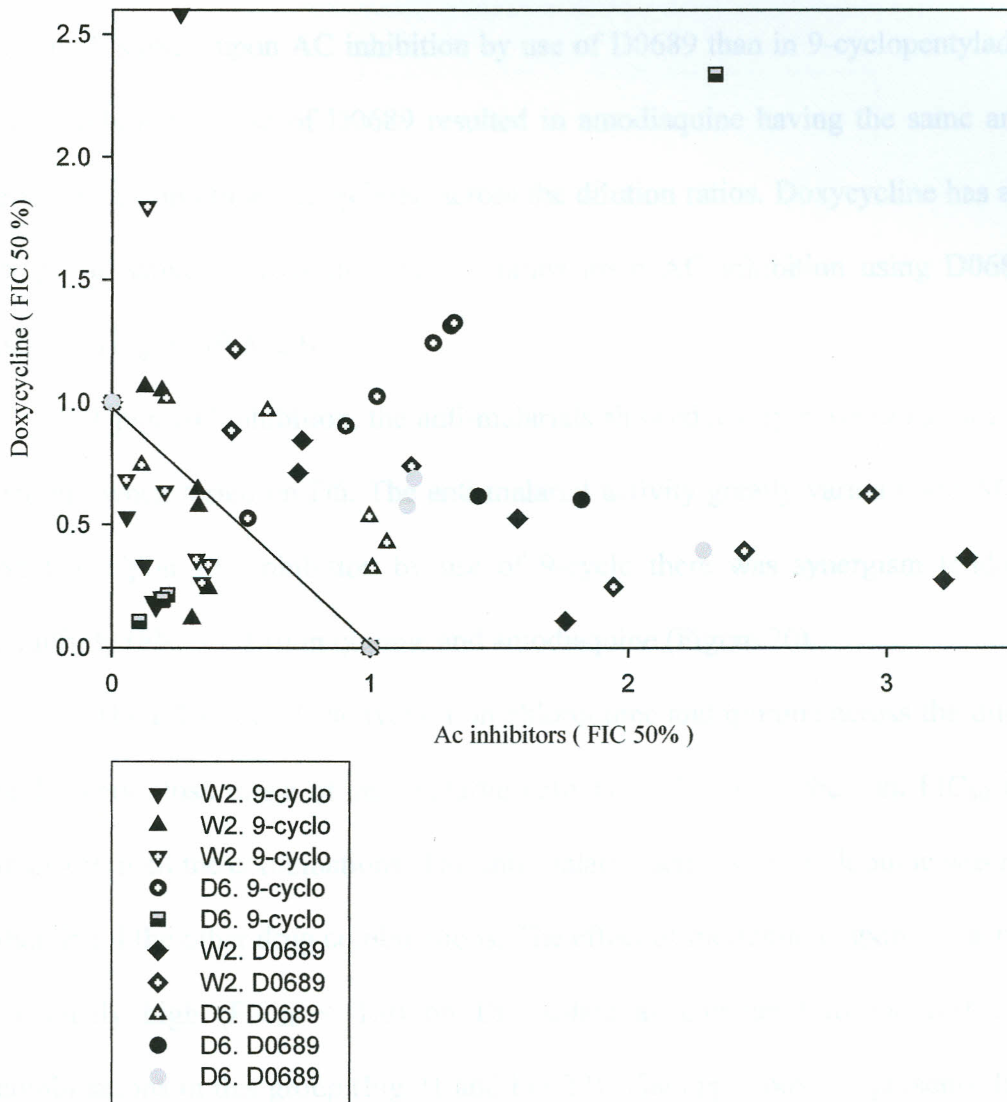


Figure 18: Adenylyl cyclase inhibitors and doxycycline FIC_{50} at various concentration ratios

3.4 Box plots representing the activity exhibited by the drug combinations across the concentration ratios.

There is variation of sum FIC_{50} across the dilution ratios in the drug combinations used in this study. This is illustrated in Fig 19 and Fig 20. Chloroquine and quinine have the same anti-malarial effect upon AC inhibition. Quinine has a notably higher anti-malarial activity upon AC inhibition by use of D0689 than in 9-cyclopentyladenine. The AC inhibition by use of D0689 resulted in amodiaquine having the same anti-malarial effect as chloroquine and quinine across the dilution ratios. Doxycycline has a very wide range of activity across the dilution ratios upon AC inhibition using D0689 on both isolates (Figure 19 & 20).

Upon AC inhibition, the anti-malarials showed a very close range of anti-malarial activity when tested on D6. The anti-malarial activity greatly varied upon AC inhibition on D6. Upon AC inhibition by use of 9-cyclo there was synergism tending towards additivity ($FIC_{50} > 1.0$) in quinine and amodiaquine (Figure 20).

The effect of AC activation on chloroquine and quinine across the dilution ratios had a very close range of anti-malarial activity on W2 with the sum FIC_{50} was greater than 1.0 in all the combinations. The anti-malarial activity of mefloquine was much lower than in all the other drug combinations. The effect of mefloquine upon AC activation was extremely high ($FIC_{50} < 1.0$) on D6 isolate as compared to the rest of the drug combinations in this group (Fig 21 and Fig 22). The upper boxes represents the deviation points above the median while the lower boxes represents the deviation points below the median (Figure 19 to 22).

Figure 19 represents the means of sum FIC_{50} of Anti-malarial-AC inhibitors on W2. The boxes represent the interquartile range in each drug combination. Short bars indicate the closeness of the sum FIC_{50} across the concentration ratios.

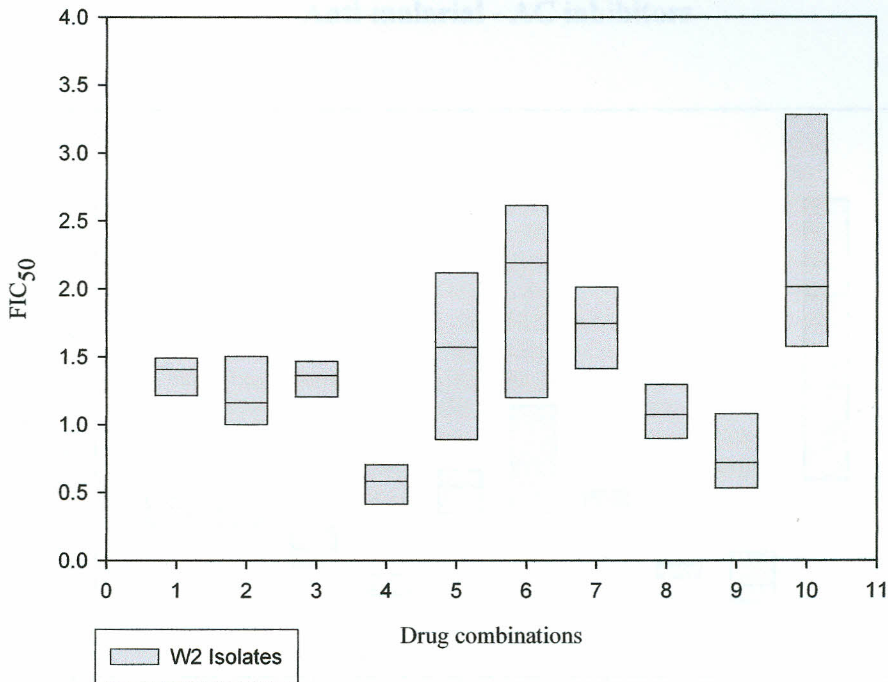


Figure 19: Anti-malarial –AC inhibitor combination assays on W2 isolates

Legend:
1 - CQN-9-cyclo
2 – CQN-D0689
3 - QN-9-cyclo
4 – QN- D0689
5 – MQN-9-cyclo
6 – MQN-D0689
7 – AQN-9-cyclo
8 – AQN – D0689
9 – Doxy-9-cyclo
10 – Doxy-D0689

Figure 20 represents the means of sum FIC_{50} of Anti-malarial-AC inhibitors on W2. The boxes represent the interquartile range in each drug combination. Short bars indicate the closeness of the sum FIC_{50} across the concentration ratios.

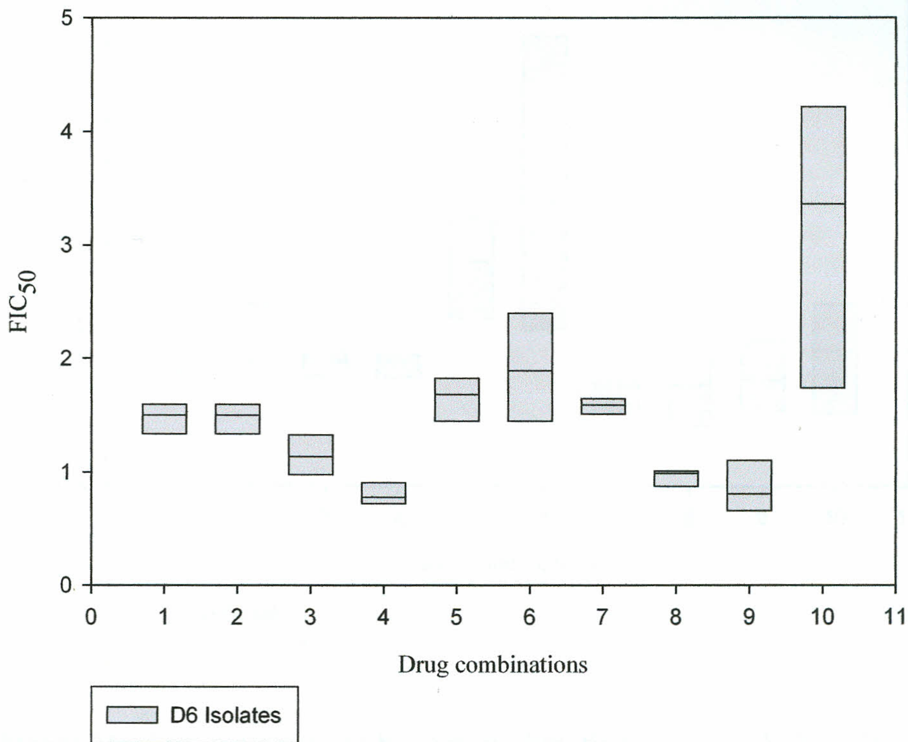


Figure 20: Anti-malarial –AC inhibitor combination assays on D6 isolates

Legend: 1 - CQN-9-cyclo
2 – CQN-D0689
3 - QN-9-cyclo
4 – QN- D0689
5 – MQN-9-cyclo
6 – MQN-D0689
7 – AQN-9-cyclo
8 – AQN – D0689
9 – Doxy-9-cyclo
10 – Doxy-D0689

Figure 21 represents the means of sum FIC_{50} of Anti-malarial-activators on W2. The boxes represent the interquartile range in each drug combination. Short bars indicate the closeness of the sum FIC_{50} across the concentration ratios.

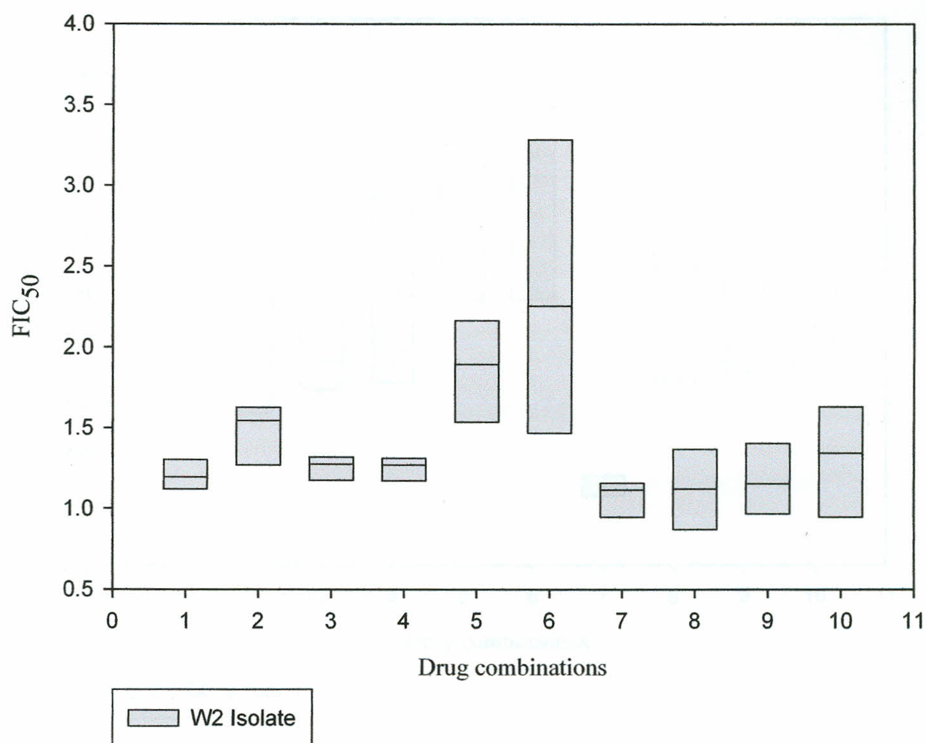


Figure 21: Anti-malarial –activators combination assays on W2 isolates

Legend:
1 - CQN-Forskolin
2 – CQN-A 166
3 - QN-Forskolin
4 – QN- A 166
5 – MQN-Forskolin
6 – MQN- A 166
7 – AQN-Forskolin
8 – AQN – A 166
9 – Doxy-Forskolin
10 – Doxy-A 166

Figure 22 represents the means of sum FIC_{50} of Anti-malarial-activators on D6. The boxes represent the interquartile range in each drug combination. Short bars indicate the closeness of the sum FIC_{50} across the concentration ratios.

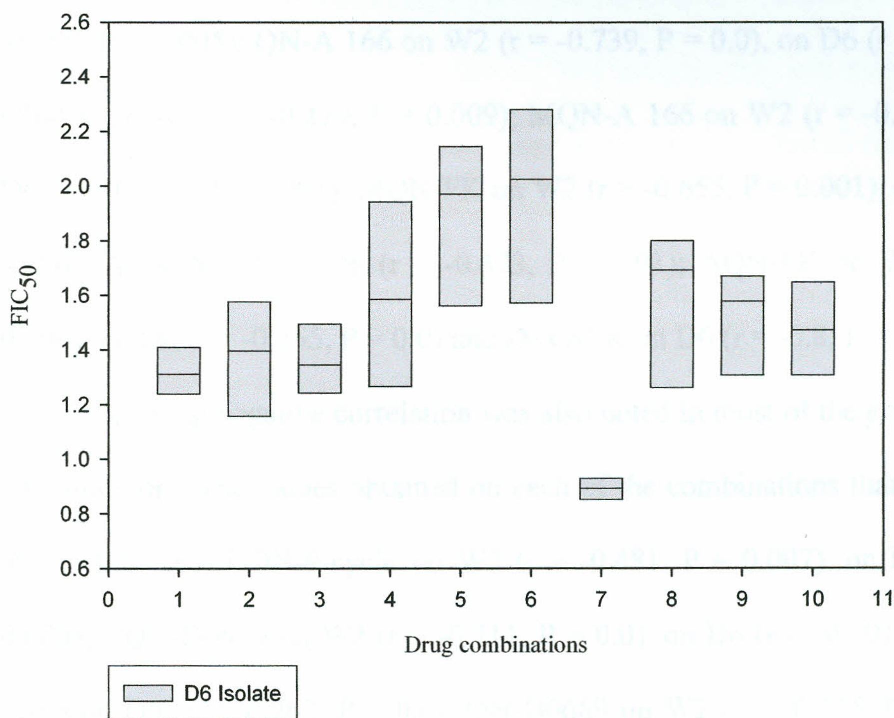


Figure 22: Anti-malarial –activators combination assays on D6 isolates

Legend:
1 - CQN-Forskolin
2 – CQN-A 166
3 - QN-Forskolin
4 – QN- A 166
5 – MQN-Forskolin
6 – MQN- A 166
7 – AQN-Forskolin
8 – AQN – A 166
9 – Doxy-Forskolin
10 – Doxy-A 166

3.5 The association in the cAMP modulators on anti-malarial activity

To find the association between the 2 drugs used in each combination, the FIC_{50} of each drug was used to compute Pearson's correlation coefficient. Most of the drugs used in the combinations showed a strong negative correlation at $P < 0.01$. The anti-malarials that showed correlation with AC and PKA activators are: CQN-FK on W2 (r = -0.509, P = 0.005); QN-A 166 on W2 (r = -0.739, P = 0.0), on D6 (r = -0.53, P = 0.003); QN-FK on W2 (r = -0.479, P = 0.009); MQN-A 166 on W2 (r = -0.642, P = 0.001), on D6 (r = -0.539, P = 0.003); MQN-FK on W2 (r = -0.655, P = 0.001), on D6 (r = -0.645, P = 0.0); AQN-A 166 on D6 (r = -0.473, P = 0.01); AQN-FK on W2 (r = -0.518, P = 0.004), on D6 (r = -0.755, P = 0.0) and Doxy-FK on D6 (r = -0.8, P = 0.0).

A strong negative correlation was also noted in most of the anti-malarials with the AC inhibitors. The values obtained on each of the combinations that showed correlation at $P < 0.01$ are: CQN-9-cyclo on W2 (r = -0.481, P = 0.007), on D6 (r = -0.477, P = 0.008); CQN-D0689 on W2 (r = -0.711, P = 0.0), on D6 (r = -0.691, P = 0.001); QN-9-cyclo on D6 (r = -0.762, P = 0.0); QN-D0689 on W2 (r = -0.535, P = 0.002); MQN-9-cyclo on D6 (r = -0.619, P = 0.004); MQN-D0689 on W2 (r = -0.761, P = 0.0), on D6 (r = –0.692, P = 0.001); AQN-9-cyclo on W2 (r = -0.667, P = 0.0), Doxy-9-cyclo on W2 (r = -0.629, P = 0.0), on D6 (r = -0.527, P = 0.003) and Doxy-D0689 on W2 (r = -0.83, P = 0.0), on D6 (r = - 0.692, P = 0.0).

The strongest correlation was observed in doxycycline with forskolin and D0689 (0.8) whereas the weakest was observed in amodiaquine with 9-cyclo on W2 (0.41). However at $P < 0.05$ there was correlation in: QN-9-cyco on W2 (r =-0.436, P = 0.016), Doxy-A 166 on D6 (r = 0.415, P = 0.023), Doxy-FK on W2 (r = -0.371, P = 0.023),

AQN-9-cyclo on D6 ($r = -0.417$, $P = 0.022$) and AQN-A 166 on W2 ($r = -0.579$, $P = -0.024$).

Table 6 represents the degree of association and the level of significance of the AC inhibitors and AC activators between isolates in the same combination. There was a strong positive correlation in Doxy-9-cyclo, Doxy-D0689, AQN- FK, AQN-9-cyclo, MQN-D0689,QN-D0689 and AQN-FK combinations. The means of sum FIC_{50} of the D6 isolate increased or decreased as that of W2 isolate. The anti-malarial activity of the combinations on D6 isolate was therefore directly proportional to that of W2 isolate. All the cAMP modulators correlated ($P < 0.05$) in all combinations except for MQN-9-cyclo, AQN-D0689 and Doxy-FK.

Table 6: Correlation coefficient between isolates (D6 & W2)

Test drug	Pearson Correlation	level of significance
CQN-FK	0.603	0.001
CQN-9-cyclo	0.825	0.001
CQN-D0689	0.69	0.001
MQN-FK	0.661	0.0
*MQN-9-cyclo	0.237	0.314
MQN-D0689	0.891	0.0
QN-FK	0.487	0.007
QN-9-cyclo	0.604	0.001
QN-D0689	0.638	0.0
AQN-FK	0.932	0.0
AQN-9-cyclo	0.887	0.0
*AQN-D0689	0.285	0.304
*Doxy-FK	0.35	0.063
Doxy-9-cyclo	0.953	0.0
Doxy-D0689	0.65	0.0

Legend - * illustrates the combinations where there was no correlation (P > 0.05)

Pearson's correlation coefficient was performed to establish the association resulting from the effect of AC activator (forskolin) on anti-malarials and that of AC inhibitors on anti-malarials when tested against W2 and D6. Table 7 represents the degree of these associations. There was a strong correlation resulting from the effect of the AC activator and AC inhibitors on anti-malarials in most of the combinations.

Table 7: Correlation coefficient between AC activators and AC inhibitors

Test drug	Isolate	Pearson's Correlation	Level of significance	Isolate	Pearson's Correlation	Level of significance
CQN-FK / CQN-9-cyclo	W2	0.577	0.001	D6	0.63	0.0
CQN-FK / CQN-D0689	W2	0.669	0.0	D6	0.798	0.0
MQN-FK / MQN-9-cyclo	W2	0.599	0.005	D6	0.856	0.0
MQN-FK / MQN-D0689	W2	0.912	0.0	D6	0.589	0.006
QN-FK / QN-9-cyclo	W2	0.671	0.0	D6	0.545	0.005
QN-FK / QN-D0689	W2	0.672	0.0	D6	0.561	0.002
AQN-FK / AQN-9-cyclo	W2	0.343	*0.069	D6	0.367	*0.11
AQN-FK / AQN-D0689	W2	0.57	0.27	D6	0.186	*0.433
DOXY-FK / CQN-9-cyclo	W2	0.588	0.001	D6	0.356	*0.053
DOXY-FK / CQN-D0689	W2	0.567	0.001	D6	-0.084	*0.67

Legend: * represents the combinations which have no correlation ($P > 0.05$)

CHAPTER FOUR

4. Discussion, Conclusions and Recommendations

4.1 Discussion

This study clearly showed that the anti-malarial activity of cAMP modulators has no anti-malarial activity that could be considered to be pharmacologically significant. The two reference strains of *P. falciparum* that were exposed to these modulators obtained very high values of IC_{50} with all the values above 10,000 ng/ml and therefore are disqualified as effective malaria chemotherapeutic agents.

In-vitro drug combination sensitivity screening is used to predict the drug combination that would be of clinical importance (Ohrt et al., 2002). Ohrt and colleagues (2002) noted that, if synergy was found *in-vitro*, then 50 % of each of the component should achieve 100 % curative rates. If antagonistic effects were found, then more that 50% of each component would be required to achieve 100 % curative rates. One aim of combination therapy is to achieve maximum curative effect of the drugs with minimal cost and drug toxicity. This paradigm disqualifies components showing antagonism *in-vitro* as candidates for combination therapy.

The paradigm for combination therapy rescued atovaquone as an anti-malarial and is now in use as atovaquone-proguanil combination (Canfield *et al*., 1995). A field study was done on the basis of the same paradigm and established that azithromycin augmented the efficacy of chloroquine and quinine (Ohrt *et al*., 2002). This paradigm was the basis of this study with an aim of establishing the interaction value of reduced and increased cAMP intracellular levels to the selected anti-malarials.

Combination therapy has been found to be a very effective way to overcome drug resistance and has been successfully used in treatment of tuberculosis and Human Immuno Deficiency Virus among other diseases (White, 1997). *In vitro* sensitivity drug screening is used as a tool to predict the combinations that would be clinically useful. If it showed that some combinations exhibited synergism and additivity, the drugs used in the combination could be pharmacologically useful. Several anti-malarials have been used effectively in combination therapy, example, artemether-lumefantrine (Coartem) that has a high efficacy and tolerance profile (Bloland, 2001).

The AC inhibitors used in this study act via P-site ligand (Johnson *et al.*, 1997). Upon inhibition of AC, there was reduced cAMP synthesis via this pathway dependent on the level of inhibition. Anti-malarials in combination with AC inhibitors exhibited a range of effects at several concentration ratios. Some of these concentration ratios demonstrated synergism and additivity, which qualified them to be considered of significance in the combination assays.

The reduction of intra-cellular cAMP generally interfered with its role in growth and development of cells. This resulted in interference of the growth and development of the host cells and the parasite. It was demonstrated that malaria infection (*P. berghei*) increased the levels of cAMP in the host cell (Sheppard *et al.*, 1981). The anti-malarials and the AC inhibitors may be interfering with different mechanisms in the parasite. The principle targets for quinolones are the inhibition of heme polymerization and the food vacuole while doxycycline targets the plastid and the mitochondrion blocking protein synthesis (Foley and Leann, 1998; Meshnick, 2002; Giancarlo *et al.,* 2003). The reduced cAMP levels in the red blood cells counteracted the mechanism of the parasite resistance.

The two major mechanisms of anti-malarial resistance are; alteration of drug transport and drug-binding affinity. Chloroquine accumulation in the parasite's food vacuole is dependent on the drug binding to an intra-cellular receptor (ATP-independent), active uptake of the drug and the charged anti-malarial molecules being trapped in the food vacuole via an ATP-dependent mechanism (Macreadie *et al*., 2000). Its resistance is mainly due to reduced drug accumulation in the parasite. It may be possible to increase drug accumulation to the levels at which the chloroquine resistance becomes therapeutically insignificant.

cAMP has a series of complex signaling pathways which leads to formation of cAMP dependent ionophores or channels. This is the exit point of calcium ions and charged chloroquine molecules during efflux assisted by the proton pumps. Previous studies have shown that cAMP and calcium signaling pathways are interrelated (Beraldo *et al*., 2005).

This study showed that, chloroquine had an additive effect upon inhibition of AC at the concentration ratio of 1:5. In this regard the reduced cAMP level might have interfered with cAMP signaling pathway and counteracted the mechanism of the parasite resistance to chloroquine. The activity of the ion channels and ionophores was reduced resulting in less drug efflux. This enabled the ATP-independent receptors to trap more drug molecules thus increased levels of the anti-malarial in the parasite.

Amodiaquine is a 4-aminoquinolone anti-malarial with a similar structure to chloroquine and shares a common pharmacological target but is a superior anti-malarial. A study done previously showed that the accumulation of chloroquine in the parasite greatly depended on the energy and the trans-membrane pH gradient in the parasite while

the accumulation of amodiaquine was not only energy and pH dependent but also showed high intra-parasitic binding sites (Hawley *et al*., 1996). The host cell pH is normally at 7.4 while the *P. falciparum* food vacuole is weakly acidic.

Amodiaquine had an antagonistic effect upon AC inhibition. This study demonstrated that its activity was greatly enhanced by AC activation. The effects ranged from synergism to additivity with only a few concentration ratios with antagonistic effect. The increased intra-cellular cAMP activated the ion channels resulting in an increased drug accumulation in the cells. The mode of action of amodiaquine is by inhibiting the parasite–specific mechanism of hemoglobin degradation (Giancarlo *et al*., 2003).

The accumulation of 4-aminoquinolone in the parasite highly depends on the pH gradient between the parasite and its environs. Chloroquine acts best at the pH of 5.4 and the activation of AC resulted in an increased level of cAMP, which is a weak base in nature. Its increased level in the cell would change the pH gradient as well as the pH at which chloroquine may work best (acidic). On the contrary, the increase in the gradient may be good for amodiaquine, which is a weaker base resulting in an increase in the pH gradient (Hawley *et al*., 1996). This would lead to more drug accumulation in the acidic food vacuole. It has been established that the activity of these anti-malarials is dependent on the drug accumulation in the parasite. This explains the difference in the activity of the two 4-aminoquinolones upon inhibition and activation of AC.

The entire quinine-AC inhibitors assays demonstrated additive to synergistic effect in all of the concentration ratios. Quinine acts on the parasites by degranulation of hemozoin, resulting in heme, which is a toxic compound to the parasite (Giancarlo *et al*., 2003). The reduced cAMP levels in the host cell counteracted the parasite's mechanism

of resistance to quinine. This is shown by the lower values of FIC_{50} obtained upon AC inhibition. The highest concentration ratio (1:5) of the inhibition seems to be the most effective for this combination.

Mefloquine targets the food vacuole and causes degranulation of the hemozoin (Foley and Leann, 1998). Mefloquine-cAMP modulators combination assays were greatly exhibited to be antagonistic. Mefloquine-9-cyclo combination assay on W2 strain of *P. falciparum* at concentration ratios of 1:4 and 1:5 showed an additive effect. This combination is not suitable for pharmacological consideration.

Doxycycline is a slow acting antibiotic with effective anti-malarial activity. doxycycline-AC inhibitors combination assays mostly exhibited synergism with 9-cyclopentyladenine in all concentration ratios but one (3:1) on both strains of *P. falciparum*. D0689 is a P-site inhibitor of AC and 9-cyclopentyladenine is non-competitive and has a lower inhibitory effect than D0689 (Desaubry and Johnsons, 1996; Johnson *et al.*, 1997). The implication is that D0689 in a better AC inhibitor. This could partially explain the lower FIC_{50} of D0689 as compared to 9-cyclo when in combination with the other anti-malarials.

9-cyclopentyladenine, on the other hand, demonstrated a greater anti-malarial activity when in combination with doxycycline as compared to D0689. In spite of their low anti-malarial activity, the AC inhibitors augmented the efficacy of doxycycline effectively in most of the concentration ratios.

Anti-malarials in combination with the AC activator revealed a general picture of antagonism. A previous study showed that mature murine red blood cells had relatively lower levels of cAMP than the other cells in circulation. However, upon malaria infection

the cAMP levels were drastically increased in the red blood cells but there was no change of cAMP levels in the other cells was detected. Parasites contain little or no AC activity but are directly or indirectly responsible for the increased levels of the host cell enzymes (AC activities are increased after infection). The increased cAMP levels are favorable for several glycolytic-based activities in the parasite. This is because several glycolytic enzymes of the parasites are regulated by cAMP dependent proteases. This is to match up with the increased demand for nutrients and minerals in an infected host cell.

In cases of infection with *Vibro cholerae* and other related enterobacteriaceae microorganisms, the enterotoxins bind to a receptor on the intestinal cell membrane while the other side binds on the AC. This activates the AC resulting in the increased production of the cAMP. The increased levels of cAMP stimulate the cells to actively secrete ions into the intestinal lumen (Bailey and Scotts, 2002).

The increased level of cAMP not only promotes growth and development of cells but also increases cAMP dependent signaling in the cells. Calcium is required by the parasite for the regulation of its cell cycle and is known to be more effective in the presence of cAMP. There is evidence showing that calcium storage by the food vacuole is linked directly to the parasite signaling and asexual development (Biagini *et al.*, 2003). The red blood cell plasma membrane contains a calcium ATPase for pumping the calcium across to the intracellular environs. Upon infection the enzyme pumps the calcium ions in the parasite allowing a calcium gradient to form. This produces an environment that makes Ca^{+2} signaling within the parasite possible (Camacho, 2003).

The increase in cAMP results in the release of Ca^{+2} from the endoplasmic reticulum and activation of a Ca^{+2} influx pathway (Beraldo *et al.*, 2005). Most of the anti-

malarials are charged, a necessity for their transportation into the parasite. The reduced drug accumulation is a major mechanism of resistance in anti-malarials due to drug efflux. The increased influx of ions (H^+, Ca^{2+} and Na^+) in the parasite may result in increased efflux of the charged anti-malarials to the environs. This may explain the general high FIC_{50} values obtained by anti-malarial- AC activator and anti-malarial – PKA activator combinations. This was absent in amodiaquine and doxycycline against W2 at some concentration ratios.

A 166 is a potent membrane permeable activator of cAMP dependent Protein Kinase I and II that mimics the effects of cAMP (Scholubbers *et al.*, 1984). Anti-malarials-A166 combination assays have greatly exhibited antagonistic effect. Amodiaquine and doxycycline showed synergism and additivity in a few concentration ratios on W2.

In this study, the cAMP modulation was shown at different levels depending on the dilution concentration ratio. The inhibition of AC at 1:5 concentration ratios (1 part of the anti-malarial and 5 parts of the inhibitor) generally exhibited the most desirable effect in this study with all the sum FIC_{50} values below 1. AQN-9-cyclo and doxy-D0689 combination assays were the only exception with FIC_{50} greater than 1.

There was a notable difference in the anti-malarial activity upon inhibition and activation of AC. It is shown that AC inhibition augmented the efficacy of some of the anti-malarials used. On the other hand, AC activation favored the parasites in all anti-malarials but amodiaquine. The evidence that the invasion of the host red blood cell by merozoites is regulated by the host β 2-adrenergic receptor, Gα subunit and cAMP can explain this. Host signaling pathways and cAMP are thought to influence the parasite

development. It has been demonstrated that antagonists for adenosine receptors blocked malaria infection of *P. falciparum in vitro* (Harrison, 2003).

The activity of anti-malarials correlated with the cAMP modulators used in most of the combination assays. The activity of anti-malarials upon activation had a strong correlation with the activity of the same anti-malarials upon inhibition on the W2 isolate. However, when tested on D6, the activity of amodiaquine and doxycycline didn't have any correlation. This was an indication that most of the cAMP modulators had related positively with one another when used in combination with anti-malarials.

The distinct biochemical properties of the cyclic nucleotide signaling pathways make them potential targets for novel chemotherapeutic strategies against malaria (Baker, 2004). This study clearly showed that AC inhibitors improved the efficacy of quinine at all the concentration ratios used.

4.2 Conclusions

The cAMP modulators have no anti-malarial activity as single agents; however, they augment the efficacy of some of the selected conventional anti-malarials. There was synergy to additive effect on quinine upon inhibition of AC in all concentration ratios. Antagonistic effect was demonstrated on all anti-malarials but amodiaquine upon activation of AC. Amodiaquine exhibited synergism to additivity upon AC activation but exhibited antagonism upon inhibition of AC. The other combinations show a wide range of effect from additivity at some drug concentrations to antagonistic effects while activation of enzymes revealed antagonistic effect.

The value of inhibition of AC on anti-malarials was not restricted to a particular strain of *P. falciparum*. The different strains responded the same to the anti-malarial

activity. The effect of AC inhibition / activation depended on the mode of action of the anti-malarials and the mechanism of resistance.

There was a very strong negative correlation between the anti-malarials and the cAMP modulators in most of the combination assays. Doxy-A 166 on D6 combination, which had a positive correlation while CQN-A 166 on D6, CQN-FK on D6, QN-FK on D6, QN-D0689 on D6, Doxy-A 166 on W2 and MQN-9-cyclo on W2 didn't show any correlation. AC activators had a strong positive correlation with AC inhibitors between the isolates except for doxycycline and amodiaquine combinations on D6 and AQN-9-cyclo on W2 and between the isolates in most of the combinations.

There was no significant difference in the activity of anti-malarials against chloroquine sensitive strain (D6) and chloroquine resistant strain (W2) but with a few exceptions. The anti-malarials and the cAMP interfered with separate functions within the parasites. The increase or decrease of the intracellular cAMP may have interfered with the mechanism of the anti-malarial resistance.

4.3 Recommendations

More studies should be done on the effect of quinine and doxycycline upon inhibition of AC at higher anti-malarial: cAMP modulator ratio. Doxycycline is a slow acting drug. In the studies done previously, the parasites were exposed to doxycycline for atleast 48 hours unlike in this study. In the future studies the parasites should be exposed for 48 hours so as to establish if the duration of exposure will have an effect on the efficacy of the anti-malarial upon the inhibition of AC. Other multi-drug resistant isolates should also be tested against QN-AC inhibitors combinations. The best drug combination(s) should be evaluated for future malaria treatment.

Studies to establish the effects of these AC inhibitors and the risk benefit analysis of these combinations should be done. *In-vivo* studies to determine the chemotherapy suitability of these combinations (QN-AC inhibitors) in animals and the pharmacological interactions of these drugs should also be evaluated.

More *in-vitro* combination studies should be done using other AC inhibitors to establish their effect on the anti-malarials. Several resistant strains of *P. falciparum* should be used in future studies to establish the variability amongst these strains. The intra-cellular cAMP should be determined upon AC inhibition and AC activation.

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APPENDICES

APPENDIX 1: Protocol for culture media preparation

Preparation of stock media

a) 900ml of distilled de-ionized water was put in cleaned glassware and 5.94 grams of Hepes added.

b) 10.4 grams of RPMI 1640 powder was then put and de-ionized water added to measure 1 litre.

c) A well rinsed magnetic bar was put in the cylinder and stirred using a magnetic stirrer at medium speed until it completely dissolved.

d) It was then passed through a 0.2µm filter unit.

Medium with 10 % Serum

a) The serum was thawed at 37 °c water bath for 20-30 minutes.

b) For every 100ml final volume the following volumes were mixed; 87.5ml of sterile RPMI 1640, 2.5ml of sterile $NaHCO_3$ and 10ml of pooled serum (equal volumes of A, B and O).

c) The media was then liquated in smaller quantities (in $25cc^3$ flasks) and flushed with 3 % CO_2, 5 % O_2 and 91 % N_2.

d) They were labeled and stored in the refrigeration temperature of between 2 °C – 5°C.

Preparation of 50 % HCT red blood cells

a) 30ml of blood was collected in ACD labeled and stored at 2 °C- 5 °C.

b) The desired volume of blood was put in a sterile 15 ml centrifuge tube(s).

c) It was centrifuged at 3000 rpm for 10 min. at 4 °C – 10 °C.

e) The supernatant fluid was aspirated and the top 2 mm of cell layer into vacuum trap.

f) The red blood cells were then washed twice (Steps c & d were repeated) using double volume of the wash to the packed cells.

g) Equal volume of the wash medium was added to the packed cells and gently mixed.

h) Each tube was labeled and stored at 2 °C- 5 °C

APPENDIX 2: Tables of results for FIC_{50} of individual drugs in combination

Table 9: FIC$_{50}$ of chloroquine in combination with the AC & PKA activators

Dilution ratio	CQN-A 166 W2		CQN-A 166 D6		CQN-FK W2		CQN-FK D6	
	Drug A	Drug B	Drug A	Drug B	Drug A	Drug B	Drug A	Drug B
1.1	0.207	1.236	0.22	1.547	0.1	1.275	0.28	1.255
	0.225	1.026	0.21	1.221	0.036	0.972	0.077	1.08
	0.151	1.616	0.187	1.993	0.136	1.066	0.087	1.544
	0.15	1.386	0.172	1.937	0.072	0.894	0.082	1.336
	0.158	1.3765	0.028	1.365	0.03	1.23	0.058	0.815
			0.026	1.094	0.037	1.044	0.035	1.222
1.3	0.612	1.221	0.541	1.267	0.276	1.173	0.752	1.125
	0.577	0.877	0.481	0.933	0.102	0.923	0.222	1.041
	0.434	1.544	0.379	1.343	0.347	0.904	0.249	1.476
	0.551	1.696	0.382	1.434	0.224	0.929	0.229	1.248
	0.357	1.2885	0.074	1.217	0.077	1.049	0.155	0.732
			0.08	1.147	0.102	0.972	0.121	1.435
3.1	0.084	1.51	0.075	1.581	0.033	1.269	0.115	1.548
	0.092	1.257	0.069	1.204	0.012	1.012	0.283	1.19
	0.049	1.561	0.044	1.414	0.056	1.302	0.03	1.581
	0.058	1.605	0.045	1.522	0.03	1.13	0.029	1.42
	0.055	1.5167	0.007	1.046	0.01	1.195	0.019	0.823
			0.008	1.049	0.014	1.186	0.012	1.299
1.4	0.64	0.957	0.504	0.886	0.359	1.144	0.907	1.009
	0.617	0.703	0.58	0.843	0.155	1.05	0.019	0.996
	0.482	1.285	0.41	1.09	0.553	1.083	0.24	1.07
	0.494	1.14	0.564	1.601	0.333	1.034	0.274	1.121
	0.413	1.0063	0.088	1.091	0.091	0.936	0.181	0.641
			0.089	0.951	0.153	1.092	0.139	1.235
4.1	0.058	1.384	0.044	1.248	0.025	1.267	0.085	1.519
	0.063	1.143	0.047	1.089	0.012	1.255	0.261	1.078
	0.031	1.319	0.03	1.263	0.044	1.379	0.021	1.483
	0.032	1.165	0.035	1.563	0.026	1.284	0.02	1.276
	0.037	1.3073	0.005	0.91	0.011	1.235	0.009	1.319
1.5	0.549	0.657	0.461	0.649	0.341	0.87	0.89	0.796
	0.554	0.505	0.418	0.486	0.163	0.885		0.734
	0.403	0.86	0.3	0.638	0.41	0.642	0.212	0.755
	0.441	0.814	0.359	0.808	0.268	0.666	0.223	0.728
	0.307	0.6603	0.078	0.773	0.112	0.915	0.171	0.484
			0.083	0.713	0.143	0.815	0.154	1.092

Table 10: FIC$_{50}$ of quinine in combination with the AC & PKA activators

Dilution ratio	QN-A 166 W2		QN-A 166 D6		QN-FK W2		QN-FK D6	
	Drug A	Drug B	Drug A	Drug B	Drug A	Drug B	Drug A	Drug B
1.1	0.147	1.142	0.157	1.533	0.089	1.002	0.08	0.981
	0.152	1.01	0.164	1.759	0.102	1.2	0.101	1.227
	0.2	1.135	0.688	1.379	0.08	0.981	0.082	1.177
	0.235	1.173	0.642	2.007	0.159	1.14	0.059	1.286
	0.138	1.098	0.19	1.289	0.092	1.287	0.097	0.91
	0.256	1.169	0.208	1.643	0.079	1.045	0.22	2.354
1.3	0.346	0.895	0.345	1.125	0.26	0.979	0.237	0.972
	0.332	0.735	0.396	1.415	0.284	1.117	0.215	0.87
	0.538	1.018	0.783	0.523	0.237	0.972	0.218	1.05
	0.491	0.817	0.953	0.994	0.483	1.157	0.158	1.15
	0.343	0.912	0.412	0.933	0.247	1.149	0.293	0.92
	0.57	0.868	0.515	1.355	0.213	0.944	0.462	1.644
3.1	0.056	1.294	0.053	1.559	0.03	1.029	0.039	1.448
	0.052	1.042	0.061	1.95	0.04	1.4	0.081	2.951
	0.076	1.298	0.321	1.927	0.039	1.448	0.03	1.298
	0.086	1.284	0.272	2.548	0.054	1.169	0.02	1.305
	0.047	1.13	0.066	1.351	0.03	1.252	0.036	1.008
	0.091	1.241	0.063	1.486	0.029	1.147	0.059	1.879
1.4	0.419	0.811	0.379	0.927	0.264	0.746	0.291	0.895
	0.394	0.654	0.444	1.188	0.369	1.088	0.307	0.932
	0.509	0.722	0.771	0.386	0.291	0.95	0.31	1.117
	0.505	0.63	0.951	0.744	0.516	0.926	0.208	1.137
	0.414	0.826	0.475	0.808	0.299	1.045	0.348	0.821
	0.679	0.775	0.45	0.887	0.319	1.063	0.434	1.16
4.1	0.04	1.245	0.035	1.378	0.024	1.085	0.022	1.097
	0.038	1.018	0.042	1.791	0.031	1.46	0.02	0.995
	0.054	1.235	0.143	1.149	0.022	1.097	0.021	1.208
	0.056	1.122	0.176	2.205	0.048	1.364	0.013	1.099
	0.077	1.411	0.037	1.159	0.022	1.154	0.054	2.317
	0.395	0.611	0.22	0.429	0.267	0.604	0.19	0.467
1.5	0.346	0.459	0.226	0.484	0.391	0.923	0.303	0.737
	0.393	0.446	0.747	0.3	0.19	0.467	0.228	0.657
	0.475	0.475	0.663	0.415	0.6	0.861	0.204	0.893
	0.396	0.631	0.398	0.541	0.306	0.856	0.312	0.589
	0.758	0.692	0.285	0.449	0.259	0.691	0.49	1.046

Table 11: FIC$_{50}$ of mefloquine in combination with the AC & PKA activators

Dilution ratio	MQN-A 166 W2		MQN-A 166 D6		MQN-FK W2		MQN-FK D6	
	Drug A	Drug B	Drug A	Drug B	Drug A	Drug B	Drug A	Drug B
1.1	0.593	1.551	0.633	1.775	0.113	2.625	0.172	2.512
	0.481	1.875	0.634	2	0.287	2.256	0.352	1.169
	0.721	2.532	0.437	0.975	0.209	1.474	0.357	1.276
	0.706	2.418	0.477	0.957	0.316	2.023	0.22	2.354
	0.742	1.623	0.529	2.125	0.303	1.592	0.287	2.256
			0.566	2.476			0.138	3.107
1.3	0.942	0.822	0.835	0.781	0.286	2.207	0.348	1.69
	0.699	0.908	0.934	0.982	0.648	1.69	0.634	0.702
	0.988	1.156	0.939	0.698	0.442	1.041	0.645	0.768
	0.994	1.135	0.83	0.555	0.525	1.121	0.462	1.644
	0.953	0.696	0.924	1.239	0.648	1.135	0.648	1.69
			0.952	1.388			0.334	2.508
3.1	0.252	1.965	0.282	2.361	0.036	2.485	0.049	2.119
	0.204	2.374	0.265	2.495	0.105	2.459	0.159	1.573
	0.377	3.943	0.139	0.927	0.078	1.639	0.144	1.535
	0.352	3.6	0.146	0.874	0.104	1.984	0.059	1.879
	0.382	2.497	0.214	2.572	0.108	1.688	0.105	2.459
			0.262	3.424			0.034	2.309
1.4	0.929	0.608	0.861	0.603	0.237	1.373	0.404	1.473
	0.662	0.645	0.955	0.753	0.667	1.319	0.672	0.558
	0.997	0.875	1.014	0.566	0.481	0.851	0.74	0.661
	1.031	0.883	0.938	0.469	0.68	1.09	0.434	1.16
	0.834	0.457	0.932	0.937	0.671	0.889	0.667	1.319
			1.1	1.203			0.313	1.767
4.1	0.196	2.047	0.159	1.783	0.021	1.909	0.038	2.238
	0.143	2.23	0.177	2.226	0.064	2.021	0.096	1.275
	0.404	5.665	0.108	0.964	0.053	1.492	0.108	1.546
	0.283	3.873	0.11	0.881	0.076	1.934	0.054	2.317
			0.202	3.54			0.02	1.795
1.5	0.68	0.356	0.418	0.235	0.156	0.721	0.288	0.84
	0.526	0.41	0.646	0.408	0.573	0.899	0.31	0.206
	0.876	0.615	1.021	0.456	0.399	0.563	0.735	0.525
	0.669	0.458	0.891	0.358	0.493	0.632	0.49	1.046
	0.675	0.296	0.674	0.542	0.715	0.754	0.573	0.899
			0.589	0.515			0.19	0.857

Table 12: FIC_{50} of amodiaquine in combination with the AC & PKA activators

Dilution ratio	AQN-A 166 W2		AQN-A 166 d6		AQN-FK W2		AQN-FK D6	
	Drug A	Drug B	Drug A	Drug B	Drug A	Drug B	Drug A	Drug B
1.1	0.242	1.118	0.196	1.174	0.172	1.056	0.122	0.86
	0.772	1.04	0.201	1.01	0.271	1.122	0.122	0.924
	0.658	1.723	0.279	1.562	0.281	0.803	0.453	0.523
			0.247	1.462	0.122	0.86	0.389	0.379
			0.694	1.709	0.122	0.924		
			0.473	1.765	0.145	0.882		
1.3	0.791	1.21	0.6	1.201	0.39	0.805	0.226	0.532
	0.989	0.444	0.591	0.989	0.686	0.952	0.276	0.689
	0.826	1.271	0.723	1.348	0.462	0.445	0.718	0.276
			0.675	1.348	0.226	0.532	0.739	0.24
			0.888	0.729	0.276	0.689		
			0.798	0.992	0.373	0.757		
3.1	0.078	1.08	0.073	1.313	0.065	1.198	0.035	0.749
	0.357	1.44	0.086	1.277	0.115	1.43	0.034	0.771
	0.318	1.861	0.115	1.918	0.139	1.226	0.23	0.797
			0.08	1.419	0.035	0.749	0.193	0.564
			0.186	1.374	0.034	0.771		
			0.147	1.641	0.056	1.017		
1.4	0.821	0.942	0.637	0.956	0.449	0.69	0.249	0.441
	0.864	0.291	0.847	1.063	0.756	0.783	0.213	0.401
	0.743	1.044	0.894	1.251	0.598	0.432	1.081	0.312
			0.772	1.139	0.249	0.441	0.8	0.195
			0.838	0.516	0.213	0.401		
			0.906	0.844	0.413	0.537		
4.1	0.056	1.027	0.051	1.216	0.052	1.282	0.03	0.844
	0.294	1.583	0.045	0.897	0.092	1.519	0.03	0.899
	0.241	1.802	0.062	1.382	0.108	1.254	0.208	0.959
			0.059	1.389	0.03	0.844	0.098	0.38
			0.103	1.54	0.043	1.048		
1.5	0.619	0.568	0.391	0.47	0.411	0.504	0.25	0.354
	0.747	0.201	0.533	0.535	0.502	0.415	0.306	0.462
	0.762	1.008	0.591	0.662	0.533	0.308	0.969	0.224
			0.431	0.508	0.25	0.354	0.717	0.139
			0.504	0.248	0.306	0.462		
			0.675	0.503	0.347	0.421		

Table 13: FIC$_{50}$ of chloroquine in combination with the AC inhibitors

Dilution ratio	CQN-9-cyclo W2		CQN-9-cyclo D6		CQN-D0689 W2		CQN-D0689 D6	
	Drug A	Drug B	Drug A	Drug B	Drug A	Drug B	Drug A	Drug B
1.1	0.152	1.416	0.122	1.206	0.531	1.288	0.795	0.743
	0.122	1.206	0.152	1.416	0.711	1.406	0.771	0.777
	0.116	1.559	0.194	1.861	0.514	1.054	0.103	1.272
	0.128	1.637	0.128	1.234	0.47	0.8	0.102	1.333
	0.109	1.578	0.143	1.514	0.323	0.685		
	0.066	1.513	0.118	1.432	0.289	0.72		
1.3	0.34	1.056	0.3	0.993	0.654	0.529	1.091	0.34
	0.3	0.993	0.34	1.056	0.745	0.491	1.249	0.419
	0.244	1.095	0.483	1.542	0.61	0.417	0.274	1.134
	0.27	1.15	0.382	1.228	0.83	0.444	0.263	1.152
	0.301	1.452	0.373	1.313	0.517	0.366		
	0.177	1.353	0.33	1.328	0.425	0.353		
3.1	0.052	0.439	0.041	1.208	0.249	1.81	0.435	1.218
	0.041	1.208	0.052	1.439	0.337	1.999	0.426	1.287
	0.035	1.399	0.057	1.63	0.222	1.363	0.045	1.679
	0.038	1.464	0.042	1.223	0.216	1.101	0.045	1.79
	0.038	1.661	0.052	1.655	0.168	1.068		
	0.022	1.516	0.043	1.546	0.137	1.023		
1.4	0.388	0.902	0.365	0.906	0.543	0.329	0.865	0.202
	0.365	0.906	0.388	0.902	0.794	0.393	0.699	0.176
	0.279	0.938	0.538	1.287	0.775	0.397	0.247	0.766
	0.326	1.043	0.453	1.091	0.767	0.326	0.272	0.895
	0.373	1.351	0.435	1.148	0.624	0.331		
	0.223	1.282	0.373	1.128	0.679	0.423		
4.1	0.033	1.244	0.031	1.24	0.129	1.248	0.271	1.014
	0.031	1.24	0.033	1.244	0.147	1.165	0.217	0.874
	0.027	1.477	0.04	1.546	0.128	1.049	0.02	1.001
	0.029	1.496	0.035	1.355	0.154	1.051	0.025	1.281
	0.027	1.547	0.04	1.702	0.133	1.129		
	0.015	1.346	0.03	1.441	0.112	1.119		
1.5	0.251	0.531	0.262	0.52	0.435	0.211	0.806	0.151
	0.262	0.52	0.286	0.531	0.785	0.311	0.534	0.108
	0.229	0.615	0.323	0.619	0.401	0.164	0.209	0.518
	0.285	0.729	0.365	0.703	0.764	0.26	0.191	0.502
	0.283	0.82	0.344	0.727	0.409	0.174		
	0.165	0.758	0.323	0.782	0.65	0.324		

Table 14: FIC$_{50}$ of quinine in combination with the AC inhibitors

Dilution ratio	Qn-9cyclo W2		Qn-9-cyclo D6		Qn-0689 W2		Qn-0689 D6	
	Drug A	Drug B	Drug A	Drug B	Drug A	Drug B	Drug A	Drug B
1.1	0.079	0.958	0.039	1.396	0.289	0.208	0.234	0.199
	0.101	1.37	0.025	1.21	0.224	0.251	0.173	0.335
	0.235	1.089	0.136	1.435	0.332	0.221	0.451	0.346
	0.311	1.25	0.161	1.436	0.262	0.24	0.484	0.494
	0.181	1.287	0.153	1.402	0.307	0.191	0.717	0.295
	0.201	1.574	0.124	1.456	0.282	0.228	0.698	0.336
1.3	0.244	0.989	0.115	0.075	0.394	0.095	0.281	0.08
	0.284	1.283	0.075	1.192	0.286	0.107	0.196	0.126
	0.493	0.762	0.37	1.3	0.383	0.085	0.569	0.146
	0.668	0.895	0.431	1.281	0.301	0.092	0.749	0.255
	0.432	1.025	0.275	0.842	0.31	0.064	0.911	0.125
	0.621	1.619	0.199	0.783	0.321	0.087	0.851	0.136
3.1	0.029	1.069		0.009	0.219	0.473	0.161	0.41
	0.035	1.415	0.009	1.345	0.184	0.617	0.132	0.768
	0.084	1.172	0.046	1.439	0.23	0.459	0.208	0.478
	0.105	1.259	0.051	1.365	0.174	0.478	0.294	0.901
	0.055	1.172	0.037	1.012	0.242	0.452	0.471	0.58
	0.074	1.746	0.025	0.89	0.197	0.478	0.446	0.643
1.4	0.267	0.813	0.163	1.46	0.277	0.05	0.296	0.063
	0.316	1.069	0.109	1.309	0.238	0.066	0.242	0.117
	0.48	0.556	0.394	1.04	0.396	0.066	0.535	0.103
	0.754	0.757	0.429	0.958	0.338	0.078	0.832	0.213
	0.531	0.945	0.354	0.812	0.341	0.053	1.029	0.106
	0.551	1.077	0.278	0.318	0.387	0.078	0.925	0.111
4.1	0.02	0.95	0.01	1.394	0.134	0.385	0.144	0.489
	0.022	1.208	0.006	1.22	0.11	0.49	0.081	0.625
	0.056	1.036	0.028	1.184	0.19	0.506	0.189	0.581
	0.079	1.264	0.032	1.135	0.146	0.534	0.199	0.811
	0.042	1.197	0.027	0.974	0.216	0.538	0.442	0.726
	0.053	1.67	0.016	0.754	0.162	0.524	0.392	0.754
1.5	0.196	0.478	0.137	0.983	0.876	0.126	0.654	0.111
	0.212	0.573	0.098	0.941	0.461	0.103	0.26	0.1
	0.429	0.397	0.293	0.618	0.743	0.099	0.553	0.085
	0.651	0.523	0.286	0.511	0.498	0.091	0.625	0.128
	0.497	0.707	0.143	0.262	0.497	0.062	0.756	0.062
	0.602	0.943	0.152	0.358	0.616	0.1	0.682	0.066

Table 15: FIC_{50} of mefloquine in combination with the AC inhibitors

Dilution ratio	MQN-9-cyclo W2		MQN—9-cyclo D6		MQN-D0689 W2		MQN-D0689 D6	
	Drug A	Drug B	Drug A	Drug B	Drug A	Drug B	Drug A	Drug B
1.1	0.244	2.27	1.008	1.565	[illegible]	0.978	0.83	3.011
	0.211	1.794	0.649	[illegible]	0.479	0.791	0.707	1.838
	0.319	1.519	0.276	1.22	0.86	1.236	0.516	0.471
	0.466	2.344	0.257	1.534	0.724	1.191	0.551	0.378
	0.254	1.454	0.224	2.39	0.525	0.274	0.712	0.28
	0.292	1.922	0.216	2.735	0.638	0.25	0.611	0.356
1.3	0.371	1.152	1.36	0.7	1.268		3.273	3.961
	0.322	0.913	1.138	0.764	1.665	0.916	2.789	2.415
	0.394	0.627	0.524	0.774	3.298	1.581	0.764	0.233
	0.507	0.844	0.53	1.056	3.543	1.943	0.343	0.079
	0.375	0.714	0.409	1.304	0.565	0.098	0.807	0.106
	0.36	0.788	0.382	1.614	0.89	0.116	0.726	0.141
3.1	0.087	2.407	0.395	1.82	0.292	1.756	0.394	4.272
	0.078	1.982	0.31		0.295	1.453	0.371	2.873
	0.131	1.865	0.098	1.29	0.619	2.657	0.21	0.572
	0.132	1.964	0.087	1.546	0.599	2.943	0.216	0.442
	0.1	1.702	0.06	1.707	0.26	0.405	0.455	0.534
	0.103	2.019	0.064	2.414	0.654	0.762	0.301	0.524
1.4	0.389	0.904	1.292	0.499	1.138	0.573	3.204	2.908
	0.319	0.68	0.95	0.47	1.655	0.683	2.762	1.794
	0.339	0.404	0.607	0.672	3.243	1.166	0.718	0.164
	0.4	0.499	0.582	0.869	3.67	1.509	0.536	0.092
	0.383	0.547	0.465	1.111	0.514	0.067	0.939	0.092
	0.351	0.577	0.455	1.439	1.26	0.123	0.69	0.101
4.1	0.057	2.12	0.237	1.465	0.312	2.511	0.323	4.685
	0.053	1.796	0.211	1.686	0.269	1.774	0.271	2.821
	0.089	1.696	0.066	1.161	0.513	2.948	0.192	0.702
	0.098	1.954	0.064	1.527	0.417	2.744	0.176	0.483
	0.074	1.698	0.043	1.658	0.267	0.556	0.37	0.582
	0.068	1.796	0.044	2.224	0.504	0.789	0.284	0.662
1.5	0.3	0.559	1.161	0.359	0.558	0.225	0.397	0.288
	0.245	0.418			0.601	0.198	0.484	0.252
	0.288	0.275	0.546	0.484	0.447	0.129	0.383	0.07
	0.302	0.302	0.349	0.417	0.628	0.207	0.394	0.054
	0.298	0.34	0.351	0.671	0.677	0.071	0.793	0.062
	0.273	0.36	0.297	0.751	1.143	0.089	0.681	0.079

Table 16: FIC_{50} of amodiaquine in combination with the AC inhibitors

Dilution ratio	AQN-9-cyclo W2		AQN-9-cyclo D6		AQN-D0689 W2		AQN-D0689 D6	
	Drug A	Drug B	Drug A	Drug B	Drug A	Drug B	Drug A	Drug B
1.1	0.253	1.343	0.141	1.651	0.888	0.377	0.843	0.175
	0.248	1.227	0.157	1.867	0.733	0.218	0.873	0.168
	0.42	2.265	0.22	1.316	0.888	0.377	0.752	0.179
	0.336	1.782	0.148	1.384	0.923	0.204	0.699	0.136
	0.216	1.259	0.888	0.377	0.965	0.216	0.852	0.176
	0.212	0.968	0.733	0.218			0.87	0.157
1.3	0.613	1.085	0.383	1.494	0.804	0.114	0.981	0.068
	0.576	0.949	0.401	1.592	0.832	0.083	0.782	0.05
	0.798	1.418	0.555	1.106	0.804	0.114	0.965	0.076
	0.784	1.382	0.534	1.683	1.018	0.075	1.124	0.073
	0.471	0.916	0.804	0.114	1.021	0.076	0.985	0.068
	0.566	0.861	0.832	0.083			0.784	0.047
3.1	0.077	1.219	0.055	1.915	0.598	0.761	0.661	0.411
	0.069	1.026	0.057	2.033	0.702	0.627	0.678	0.391
	0.215	3.474	0.089	1.595	0.598	0.761	0.527	0.375
	0.214	3.395	0.06	1.682	0.713	0.473	0.584	0.34
	0.089	1.557	0.598	0.761	0.708	0.476	0.649	0.402
	0.094	1.287	0.702	0.627			0.682	0.368
1.4	0.658	0.873	0.534	1.561	0.769	0.082	0.905	0.047
	0.615	0.761	0.498	1.483	0.744	0.055	0.955	0.046
	0.91	1.216	0.694	1.039	0.769	0.082	0.872	0.052
			0.437	1.017	0.96	0.053	0.934	0.045
	0.578	0.843	0.769	0.082	0.979	0.055	0.895	0.046
	0.429	0.49	0.744	0.055			0.956	0.043
4.1	0.054	1.152	0.043	2.006	0.529	0.897	0.569	0.471
	0.052	1.026	0.045	2.137	0.46	0.548	0.556	0.427
	0.172	3.725	0.061	1.46	0.529	0.897	0.517	0.491
	0.125	2.732	0.041	1.523	0.724	0.639	0.579	0.45
	0.066	1.547	0.529	0.897	0.76	0.68	0.589	0.486
	0.063	1.144	0.46	0.548			0.547	0.394
1.5	0.528	0.561	0.583	1.365	0.855	0.073	0.628	0.026
	0.516	0.51	0.582	1.386	0.754	0.045	0.474	0.018
	0.776	0.836	0.813	0.973	0.855	0.073	0.589	0.028
	0.911	0.966	0.722	1.348	0.817	0.036	0.701	0.03
	0.4	0.467	0.855	0.073	0.735	0.033	0.649	0.027
	0.453	0.413	0.754	0.045			0.454	0.016

Table 17: FIC$_{50}$ of doxycycline in combination with the AC inhibitors

Dilution ratio	Doxy-9-cyclo W2		Doxy-9-cyclo D6		Doxy-D0689 W2		Doxy-D0689 D6	
	Drug A	Drug B	Drug A	Drug B	Drug A	Drug B	Drug A	Drug B
1.1	0.019	0.201	1.416	1.416	1.57	0.767	0.62	0.938
	0.234	0.469	1.206	1.206	1.569	0.281	0.583	0.994
	0.189	0.297	0.21	0.21	0.898	0.572	4.033	0.333
	0.475	0.99	0.186	0.186	1.419	0.908	3.222	0.456
	0.037	0.242					2.15	0.564
	0.372	1.031					2.429	0.225
1.3	0.054	0.195	0.34	1.056	3.205	0.522	1.034	0.521
	0.269	0.18	0.3	0.993	3.41	0.204	0.958	0.545
	0.238	0.125	0.077	0.202	2.487	0.528	7.622	0.21
	0.511	0.355	0.081	0.182	3.374	0.72	6.69	0.316
	0.092	0.198					3.884	0.34
	0.563	0.521					5.369	0.166
3.1	0.075	2.448	0.052	1.439	0.698	1.024	0.195	0.885
	0.452	2.718	0.041	1.208	0.742	0.398	0.225	1.153
	0.207	0.979	0.107	2.551	0.441	0.844	1.926	0.477
	0.179	1.117	0.105	2.121	0.483	0.927	1.707	0.724
	0.12	2.331					1.017	0.8
	0.152	1.264					1.269	0.353
1.4	0.098	0.269	0.388	0.902	3.376	0.412	1.115	0.422
	0.287	0.144	0.365	0.906	3.06	0.137	1.013	0.432
	0.233	0.92	0.116	0.229	2.509	0.4	5.533	0.114
	0.438	0.228	0.119	0.2	2.389	0.382	5.377	0.19
	0.173	0.28					4.287	0.281
	0.573	0.397					4.874	0.113
4.1	0.004	0.183	0.033	1.244	0.513	1.003	0.1	0.606
	0.11	0.878	0.031	1.24	0.958	0.686	0.129	0.883
	0.128	1.065	0.006	0.195	0.464	1.184	1.585	0.523
	0.007	0.179	0.001	0.016	0.488	1.249	1.25	0.708
	0.104	1.186					0.755	0.792
							1.584	0.587
1.5	0.11	0.238	0.286	0.531	1.42	0.139	0.992	0.3
	0.232	0.093	0.262	0.52	2.092	0.075	1.025	0.349
	0.238	0.075	0.13	0.205	1.334	0.17	7.687	0.127
	0.383	0.16	0.136	0.186	2.55	0.326	3.442	0.098
	0.19	0.247					4.26	0.223
	0.506	0.281					5.892	0.109

Table 18: FIC$_{50}$ of doxycycline in combination with the AC & PKA activators

Dilution ratio	Doxy-A 166 W2		Doxy-A 166 D6		Doxy-FK W2		Doxy-FK D6	
	Drug A	Drug B	Drug A	Drug B	Drug A	Drug B	Drug A	Drug B
1.1	0.787	0.902	1.119	0.53	0.604	0.725	0.678	0.696
	0.44	0.946	1.128	0.883	0.563	0.712	0.768	0.965
	1	1.312	1.105	0.351	0.255	1.209	0.669	1.383
			0.887	0.512	0.254	1.329	0.489	1.291
					0.664	0.633	0.525	0.861
					0.698	0.613	0.483	1.16
1.3	1.152	0.439	1.222	0.193	1.002	0.401	1.011	0.345
	1.1	0.782	1.666	0.435	0.92	0.387	1.179	0.493
	1.789	0.783	1.66	0.176	0.578	0.912	1.099	0.759
			1.198	0.23	0.632	1.102	1.186	1.044
					1.09	0.346	0.862	0.471
					1.14	0.334	0.939	0.752
3.1	0.225	0.775	0.553	0.788	0.177	0.636	0.244	0.752
	0.189	1.207	0.547	1.281	0.173	0.654	0.261	0.983
	0.297	1.171	0.783	0.747	0.071	1.004	1.99	1.23
			0.443	0.767	0.076	1.19	0.171	1.355
					0.242	0.692	0.178	0.879
					0.265	0.699	0.155	1.116
1.4	1.202	0.346	1.132	0.134	1.085	0.326	1.103	0.283
	1.134	0.604	1.278	0.25	0.993	0.314	1.074	0.337
	2.067	0.678	1.492	0.119	0.619	0.732	1.204	0.622
			1.269	0.183	0.676	0.884	1.316	0.868
					0.962	0.229	1.007	0.413
					0.914	0.201	0.87	0.522
4.1	0.152	0.696	0.46	0.869	0.153	0.733	0.217	0.893
	0.148	1.259	0.429	1.353	0.135	0.683	0.2	1.005
	0.333	1.748	0.537	0.683	0.055	1.037	0.151	1.249
			0.377	0.87	0.056	1.163	0.119	1.252
					0.168	0.64	0.13	0.854
					0.193	0.68	0.118	1.137
1.5	0.935	0.214	0.593	0.056	1.074	0.258	0.971	0.199
	1.143	0.488	1	0.15	0.003	0.252	0.821	0.2006
	1.454	0.382	1.424	0.091	0.508	0.481	1.229	0.508
			0.771	0.089	0.564	0.59	1.311	0.695
					0.825	0.157	0.829	0.272
					0.949	0.167	0.659	0.316